Seroprevalence and Characteristics of Hepatitis E in the United States

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Abstract

Hepatitis E is not well-understood in the United States. Seroprevalence, transmission, and subclinical manifestations have either not been studied of been the subject of a small number of studies with limited conclusions. The purpose of this dissertation was to better understand the role of Hepatitis E in the United States. This dissertation updated the seroprevalence estimates in the United States, investigated previous inconsistencies in the seroprevalence, identified potential risk factors, and examined a potential for subclinical liver abnormalities in persons recently infected. We found seroprevalence in the United States has remained stable at 6%. In addition, we found non-Hispanic Asians had especially high seroprevalence compared to other demographic groups. We also determined the difference between seroprevalence estimates from the 1990s compared with modern seroprevalence estimates were largely, but not completely due to differences in diagnostic assays. We were unable to find any association between recent HEV infection and liver inflammation; however, we found a tenuous relationship between past HEV infection and liver fibrosis. While this paper was unable to find risk factors suggesting a causal pathway or a substantial public health impact, it does not diminish the importance of studying HEV in populations at high risk for complications – particularly immunocompromised persons.

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Introduction

Hepatitis E Background

Hepatitis E is a viral infection with a dual nature: one present in developing countries, one present in developed countries. In developing countries, Hepatitis E is characterized by large waterborne outbreaks with significant morbidity and mortality – especially in pregnant women. In developed countries, waterborne transmission of Hepatitis E leading to clinical disease is negligible to non-existent; instead, it manifests sporadically without a clear source and with low morbidity [1]. There are multiple and interrelated reasons for the manifestations to differ by country. Prime amongst them is the water supply in developed countries is more secure from pathogenic agents as compared to the water supply in developing countries. In addition to the difference in water supply, different genotypes of Hepatitis E are found in developed countries. In terms of public health impact, the predominant Hepatitis E genotype in the developing world is genotype 1, and the predominant type of Hepatitis E in the developed world is genotype 3. Genotype 2 and genotype 4 also exist but appear to be less relevant than genotypes 1 and 3 in part due to their limited geographic scope. There has been a single verified case of genotype 7 in humans [2]. Hepatitis E is not unique to humans; it has been found to infect swine, bovine [3], deer, rabbit, mongoose, chicken, camel, rat, ferret, greater bandicoot, Asian musk shrew, mink, moose, and fish; some, but not all, of these animals host human-transmissible Hepatitis E [4]. Genotypes 3 and 4 have animal hosts, but genotypes 1 and 2 are only found in humans.

Hepatitis E Virus Characteristics

The Hepatitis E virus is a single-stranded RNA virus of the *Hepeviridae* family and is genetically unrelated to the other hepatitis viruses. Like all viruses, the Hepatitis E virus consists of nucleic acids encapsulated in a protein structure called a capsid. Unlike some viruses such as Hepatitis D virus, Hepatitis E virus does not have a lipid envelope. Hepatitis E virus uses viral reproduction to replicate; viral reproduction is a process in which a virus gains access to the interior of a host cell, the viral genetic material is read by the host's metabolic components, eventually resulting in creating copies of the virus. Its genome is 7.2 kilobases (kb) and contains 3 open reading frames (ORFs) – which are DNA or RNA segments that essentially define the proteins produced. Open reading

frame 1 (*orf1*[1]) codes for a large, nonstructural protein with many functions; it is unclear if the proteins remains as a single protein or if it is cleaved into distinct functional units after translation [5]. One of the units is suspected to be a methyltransferase that caps the viral RNA – a process that assists in the creation of protein and in the evasion of triggering a host immune response [6]. A protease, a type of enzyme that cuts proteins, is also encoded by *orf1*; its role in Hepatitis E virus is unknown. A helicase is also encoded by *orf1*; helicases are essential to viral reproduction as they unwind genetic material allowing for its reproduction. This helicase is also thought to assist with the capping process done with the methyltransferase. *orf1* also encodes for an RNA dependent RNA polymerase – an essential part of any RNA virus – this protein is responsible for creating copies of the genetic material. Open reading frame 2 (*orf2*) encodes the viral capsid. The viral capsid is not just a protective casing, but also has surface molecules that can serve a variety of roles – including to enter a host cell. The surface molecules can be proteins or other molecules (e.g. carbohydrates); they can be designed as part of the capsid its self, or molecules may attach to the capsid surface. The surface of Hepatitis E virus is populated by many protein-carbohydrate complexes known as glycoproteins. A study demonstrated even slightly altering capsid of Hepatitis E virus made it unable to infect a host [7]. Characterizing the viral capsid is also important because vaccines train the immune system to recognize specific capsid elements and mark them for elimination. Because of the wide-ranging functionalities encoded by *orf2*, it has been the focus of much of the research on Hepatitis E genomics. Open reading frame 3 (*orf3*) encodes for a protein of unknown function which is predicted to be involved with extending the life of the cell, avoiding triggering an immune response and/or assisting in energy management [5]. Because the immunological assays cannot distinguish between genotypes, examining the differences in the genome sequences is the only way to establish which genotype is responsible for an infection.

Scope of Hepatitis E Genotypes

Like other viruses, Hepatitis E has multiple strains. Outside of a single aberrant case, four different genotypes of Hepatitis E are found in humans: 1, 2, 3, and 4. These genotypes are not only genetically distinct, but also have different routes of transmission,

[1] *orf1* in italics and lower case references the gene; ORF1 capitalized and not italicized references the protein. For the purpose of the discussion, the terms are mostly interchangeable.

different endemic areas, and different at-risk populations. To understand the different presentation and different public health relevance between the developing world and the developed world, it is needed to understand the difference between the genotypes.

Genotype 1: The virus now known as Hepatitis E was first implicated in a 1978 outbreak of hepatitis; in 1980, a paper hypothesized the 1978 outbreak was due to a heretofore undefined virus [8]. In 1983, immune electron microscopy identified the structure of the virus responsible for Hepatitis E [9]. In 1992, the genome of hepatitis E was sequenced from a case in Burma, identifying the genotype of what likely caused the first known outbreak of Hepatitis E [10]. This genotype has been seen in some Asian countries, African countries, and South American countries. Genotype 1 has no known animal host other than humans. Genotype 1 is the strain responsible for most large outbreaks. Like the other genotypes of Hepatitis E, the transmission route is fecal-oral. It is most often spread through a contaminated water source, but also can be spread through vertical transmission [11]. Proper sanitary habits, such as a reduction of defecation in fields can reduce the risk of an outbreak. It is not clear whether genotype 1 was previously present in developed countries then eliminated through securing the water supply or if genotype 1 has never been endemic to developed countries. In addition, because of this strain, two vaccines against Hepatitis E have been developed – though the vaccines also protect against the other genotypes [12].

Genotype 2: In 1992, a genetically distinct strain of Hepatitis E was identified in Mexico [13]. Apart from Mexico, this genotype has also been seen in some African countries. This genotype is generally mild, and few if any very severe cases have been reported [14]. Genotype 2 has no known animal host other than humans. Like genotype 1, its primary transmission path is fecal-oral and is spread through contaminated water. Due to only a few geographic areas being affected and mild disease progression, genotype 2 is not regarded as a significant public health problem.

Genotype 3: In 1997, a third genetically distinct strain of Hepatitis E was identified in the United States [15]. The strain was noted for its high similarity to strains found in swine [16]. This genotype has since been seen worldwide. Genotype 3 has been found in and potentially transmitted by swine, rabbits, mongoose [4], deer [17], and shellfish [18]. While foodborne transmission is thought to be the primary mode of transmission, working with infected swine is a potential pathway [19] [20]. Genotype 3 may have distinct, granular geographic patterns. Genotype 3 has been found in wastewater [21], but it is not known

the extent to which transmission is waterborne. Unlike the other genotypes, type 3 has been implicated in chronic Hepatitis E, particularly in immunocompromised populations. Due to its widespread nature and ability to cause chronic Hepatitis E, genotype 3 likely ranks second of Hepatitis E viruses in terms of public health impact.

Genotype 4: In 1999, a fourth genetically distinct strain of Hepatitis E was identified in China. This strain has been seen in other East Asian countries as well as a few countries in Europe. Genotype 4 has been found in and potentially transmitted by swine [4]. The potential for foodborne transmission leads to frequent comparisons to genotype 3. This genotype is not well understood; it has been suggested it may be more virulent than genotype 3 based on cell models [22] and epidemiologic data [23], but epidemiologic evidence supporting this notion may be the result of selection bias [24]. Waterborne transmission of genotype 4 is suspected, but no cases have been definitively linked to waterborne transmission. Despite being potentially more virulent than genotype 3, its localized nature and lack of outbreaks suggests it overall public health impact is low.

Symptoms and Detection

Though transmission differs greatly between genotypes, clinical presentation in symptomatic infections is uniform between genotypes. When symptomatic, Hepatitis E has an incubation period of 15-60 days with a mean of 40 days and presents similarly to other hepatotropic viruses that induce hepatic inflammation [1]. Symptoms may include fever, anorexia, arthromyalgia, pruritus, dark urine, diarrhea, nausea, headache, abdominal pain, asthenia, vomiting, purpuric rash and jaundice; these symptoms last for a month or more [25] [26] [27] [28]. During the course of a symptomatic infection, serum alanine transaminase (ALT) are sharply increased [29]. Other biochemical changes occurring during a symptomatic infection include increased aspartate aminotransferase (AST), bilirubin, alkaline phosphatase, and γ-glutamyltransferase [30]. Biochemical changes occurring in subclinical cases are not well understood. Risk factors for serious complications include pregnancy, immunocompromised status and cirrhosis [31]. Serious complications include severe liver injury, acute liver failure, perinatal mortality for pregnant women, and death [29]. The risk factors and complications vary by genotype; most notably, pregnant women have only been shown to be at greater risk when infected with genotype 1. Acute Hepatitis E has no specific treatment formally recommended [32],

but treatment with ribavirin has been highly effective in case studies and increasingly in the field as a whole [33] [34] [35].

Due to the largely non-specific symptoms and similar presentation to other Hepatitis illnesses, suspected cases must be confirmed through diagnostic testing. While no FDA-approved serological test exists, serological tests obtained from other countries are the most-used method of detecting Hepatitis E. In the recent past, in-house assays immunological assays were predominant, but now most assays are produced and marketed by companies to the institutions. There are several immunological assays currently available including, but not limited to Wantai, Euroimmun, MP, Dia.pro, DSI, Gene Labs, and Mikrogen; they all are based on detection of antibodies, not the virus itself. While there is some controversy regarding which diagnostic tests are superior [36], there are several similarities between all of them. The tests marketed now are argued to be superior than the in-house assays used previously [37]. Despite Hepatitis E having four major genotypes, all major genotypes share a single serotype which makes immunological assays able to detect all major genotypes of Hepatitis E, but unable to distinguish between them. Immunological assays are effective in detecting both temporally distant infections – IgG is 89-100% effective in detecting cases within 2 years – and temporally proximal infections – IgM can detect 73% of patients within 26 days but ~0% of patients 6-7 months after the infection is cleared [38]. Despite the high sensitivity reported for IgG in general, some IgG tests are more sensitive than others. Notably, the sensitivity of the GL-type assay, which use a mixture of peptides from ORF-2 and ORF-3, may be around 56% compared to the 98% sensitivity of the PE2-type assay, which uses structural peptides from ORF-2, despite both having similar specificities [39]. The assay primarily used in the data available is the DSI DS-EIA-ANTI-HEV-G/M assay [40]. This set of assays has high specificity in both IgM and IgG, 99% and 99% respectively, but substantially lower sensitivity, 63% and 72% respectively [41]. A key drawback of immunologic assays is the reliance on a functional immune system; immunological assays are less reliable when used in patients who are immunocompromised – this is a critical gap because Hepatitis E can manifest as chronic Hepatitis E in immunocompromised individuals, and specific protocols can be used to treat chronic Hepatitis E [32].

PCR techniques show some promise in improving detection in ongoing or recent infections, especially in immunocompromised individuals [42]. PCR techniques also have the advantage of being able to distinguish between different genotypes of Hepatitis E. Unfortunately, the quality of PCR techniques varies substantially from lab to lab [43]. Another potential disadvantage of PCR is it detects viral RNA, so PCR cannot detect recent or distant cases where the viral RNA has already been cleared – more an issue for epidemiologic studies than for clinical purposes. A weakness of all techniques is diagnostic testing cannot identify cases if it is not performed. Two studies have shown a non-insignificant number – 22% and 3% – of drug-induced liver injury cases in the developed world were actually due to Hepatitis E [44] [45]. Because testing for Hepatitis E is not consistently done in persons who may have it, incidence and prevalence estimated from clinical testing will underestimate the true incidence and prevalence of Hepatitis E.

Hepatitis E in Developing Countries

In many developing countries, Hepatitis E – genotype 1 in particular – is a major public health problem. However, Hepatitis E is not an endemic disease with a relatively stable incidence year-to-year such as malaria. Instead, Hepatitis E manifests in the form of sporadic and potentially large outbreaks – outbreaks that can exceed 100,000 cases [46]. Large outbreaks such as these are almost exclusively caused by Hepatitis E genotype 1 and mainly infects young males. These occurrences of Hepatitis E are only partially seasonal [47], and they are highly linked to the biogeography of the area [48]. The mode of transmission is typically fecal contamination of the water supply [49], but some person-to-person transmission has been reported – albeit it is still likely this was transmitted via the fecal-oral route [50]. The attack rate of this Hepatitis E is fairly high – around 50% [51].

The case-fatality rate of these Hepatitis E infections is not high – usually between 0.2% and 4%. Despite young men being more likely to be infected by genotype 1, pregnant women have exceptionally high morbidity and mortality – having a case fatality rate of 10% to 25% [52]. The reason for the much higher case fatality rate in pregnant women is not currently known. Due to large outbreaks and high mortality in vulnerable populations, two vaccines have been developed, but their impact is not currently known [12]. In addition, transmission should be able to be interrupted by ensuring water meant for

human consumption has been boiled as the Hepatitis E virus cannot withstand temperatures over 70°C [53].

The prevalence of individual genotypes in the developing world is not known because most of the testing is done through immunological testing – which cannot distinguish genotype due to the genotypes sharing a single serotype. There is some evidence that genotype 3 is rare compared to genotype 1 in places where genotype 1 is endemic. One piece of evidence is despite the widespread occurrence of Hepatitis E in developing countries, chronic Hepatitis E is extremely rare [54]. As genotype 1 cannot manifest as chronic Hepatitis E, but genotype 3 does occasionally manifest as chronic Hepatitis E this supports the hypothesis that genotype 3 is rare compared to genotype 1 in places where genotype 1 is endemic. Nevertheless, it is possible that chronic Hepatitis E is not seen in the developing world for reasons other than low prevalence of genotype 3: reports of chronic Hepatitis E could be overshadowed by reports outbreaks of Hepatitis E and/or the at-risk population – namely transplant patients – is substantially smaller in developing countries versus developed countries.

An important point is the above discussion of Hepatitis E in developing countries only applies to developing countries where genotype 1 (and to some extent genotype 2) is endemic. Many developing countries do not see Hepatitis E as a public health crisis due to the lack of genotype 1 – possibly either because they have a secure water supply or because of geographic considerations. In fact, the distinction between the developing countries and developed countries is severely undercut by a heterogeneity among the developing countries; for instance, Hepatitis E poses much less a public health problem to Latin American countries than Southeast Asian countries [55]. The distinction between developing countries and developed countries is useful in that many large developing countries has endemic genotype 1; and few, if any, developed countries have endemic genotype 1 (table 1).

Hepatitis E in Developed Countries

In developed countries, Hepatitis E has been increasingly recognized as a potential public health concern since chronic Hepatitis E has been characterized. Apart from

chronic manifestations, Hepatitis E in the developed world is generally mild. The large outbreaks and the high fatality in pregnant women that are seen in developing countries are absent in developed countries. Rather than primarily affecting young men, Hepatitis E in developed countries primarily affects middle-aged and older men. Most infections are sporadic and asymptomatic. Due to this elusive nature, determining risk factors for infections has been difficult, and risk factors may vary between countries. The only mode of transmission implicated in outbreaks in the developed world thus far has been foodborne [56], but sporadic cases are far more common than outbreaks. Transmission causing sporadic cases may be foodborne, from close contact with infected animals – including hunting [57] –, from contaminated water, or from blood transfusions. People with jobs that bring them in contact with pigs [58], including pig farmers [59] [60] and pig veterinarians [20] have been shown to be at substantially higher risk of infection than people with other occupations. Seasonality has been shown to have a minor impact in developed countries [61]. Curiously, people infected with HBV were more likely to also have been infected with HEV [62]. Cooking foods at temperatures above 70°C should prevent foodborne transmission [53].

One common feature of Hepatitis E in developed countries is the potential for chronic Hepatitis E. Chronic manifestations of Hepatitis E is a relatively newly discovered facet of the infections – it was not described in the literature until 2008 [63]. There is no formal definition of chronic Hepatitis E, but it is generally regarded as an infection lasting at least 3-6 months [64]. In cases of chronic hepatitis E, a smaller proportion of the cases are asymptomatic and cirrhosis of the liver is more common – leading to higher mortality rates [65]. Other serious complications – such as Guillain–Barre syndrome [66] and impaired kidney function [67] – may be caused by chronic hepatitis E, but further research is required. Chronic hepatitis E is most likely to occur within someone immunocompromised, including HIV infected individuals, individuals with hematological cancers, and most frequently seen in individuals with solid organ transplants. One study showed approximately 66% of solid organ transplant patients infected by Hepatitis E virus developed chronic Hepatitis E [65]. The dominant pathway for infection in these cases is not known. Blood transfusions may be a likely source in transplant cases [68], but more traditional pathways are more likely in patients who are immunocompromised for reasons other than solid organ transplants or not immunocompromised at all. The

treatment of chronic hepatitis E may differ from strictly supportive care which is traditionally used in acute Hepatitis E – either ribavirin, pegylated interferon-α, or a combination of the two are valid treatments. Pegylated interferon-α is often discouraged due to potentially serious side effects. In extreme cases, a liver transplant may be performed [32].

Like the heterogenous nature of Hepatitis E in developing countries, there appear to be substantial differences of Hepatitis E prevalence and overall impact in developed countries as well. The difference of prevalence in developed countries is not understood and may be due to factors from geography to culinary practices – especially in terms of pork consumption. For instance, most of the chronic Hepatitis E cases come from Europe and few appear elsewhere – such as the United States. The reason for this is unknown but may tie into differences in overall prevalence and patterns of infection. Because chronic Hepatitis E is a serious condition, Hepatitis E in Europe has been the subject of significantly more scholarship than Hepatitis E in the United States. Examining Hepatitis E in the United States may lead to a greater understanding of why Hepatitis E appears to be a more significant health concern in Europe than in other developed countries.

Though several studies have examined Hepatitis E in the United States, neither the prevalence nor the impact of Hepatitis E is well-understood. Previous studies have found unacceptably large ranges of possible seroprevalence and lacked either statistical power or directness in their data and research question. The aim of this project was to further characterize the prevalence of Hepatitis E, review the risk factors, examine at-risk populations, assess liver function and estimate the potential impact. The primary aim, specifically, was to assess the seroprevalence across the United States.

The two secondary aims of this study were: (i) investigate the vastly differing findings between continuous National Health and Nutrition Examination Survey (NHANES) and NHANES III using new serological testing data, (ii) examine biochemical changes in liver function in people with recent infection to better understand the mechanism for liver

damage in Hepatitis E infection and why some populations are at greater risk. This project utilizing data collected by NHANES between 2009-2016. Sera collected from NHANES III, but tested as late as 2013, will also be used.

The NHANES Study

The NHANES study is conducted by the National Center for Health Statistics, a branch of the CDC [69]. The NHANES study has been active since the 1960's. Its goal is to comprehensively assess the nutrition and health of adults and children in the United States. NHANES combines both surveys and physical examinations. One of the NHANES priorities is to create a nationally representative sample, so it provides weights to simulate the U.S. population based on their samples. In this way, the NHANES study sets the national standards for weight, height, and blood pressure. In addition, most of the data collected is released to the public for further data analysis by independent researchers. NHANES has numerous studies based on its data, with a wide range of study topics including diverse fields such as: cardiovascular disease, mental health, medication use, nutrition. This paper will primarily use serological testing for Hepatitis E, which was conducted in years 1988-1994 and 2009-present.

Dissertation Objectives

My dissertation includes three manuscripts. The first manuscript covers the primary aim of characterizing demographic characteristics, risk factors, and short-term trends in Hepatitis E. The second manuscript examines the various serological assays used on the NHANES III samples. The third manuscript examines the liver function sub-study. These three manuscripts are a much-needed updated to our current knowledge of Hepatitis E in the United States as well as contribute to a greater understanding of Hepatitis E genotype 3 in general.

Study Population

NHANES Background

This study will use the data provided by NHANES. The study will only examine data from years that included Hepatitis E serological testing. Most data will be from the continuous NHANES (years 2009-2016), but some data will be used from NHANES III (1988-1994). Prior to 1999, NHANES were numbered (I, II, and III) collected data over a span of several years, then waited several years until beginning the next set of data collection. In 1999, NHANES began continuously collecting data. Both the continuous NHANES and NHANES III are cross-sectional studies that have been used to calculate national statistics. While it is possible to calculate nationally representative statistics based on NHANES, the raw sample is not nationally representative – it oversamples minority groups to provide more accurate subgroup statistics. The methodology of sampling within the NHANES study is not consistent between studies as sampling methods and important sub-groups continue to change. Despite the changing methodology behind NHANES, the weights are similarly adjusted to ensure that different cycles of NHANES can be directly compared to one another [70]. In addition, NHANES only covers non-institutionalized civilian residents of the United States which includes resident non-citizens but excludes active duty military personnel and U.S. citizens living overseas.

NHANES Sampling Methodology

NHANES uses a 4-stage multi-cluster design: counties, segments, households and individuals. The goal of NHANES is to create a self-weighting sample within each demographic group of interest – that is, within each of the 87 demographic subgroups, every individual has the same chance of being selected –, to create a nationally-representative sample, and to be cost-effective and efficient; this three-pronged goal necessitates a complex methodology in each of its four stages. The complexity is highest in the first stage and in the fourth stage. In short, the county stage favors counties with large numbers of oversampled groups (i.e. groups that are sampled more than they would be in a simple random sample) and counties that have not been selected before; the segment stage favors census tracts with large numbers of oversampled groups; the household stage is a simple probability sample; the individual

level favors people from oversampled demographics and maximizes number of people sampled per household.

The primary sampling unit (PSU) consisted of counties – or county-equivalent regions – individually or in sets of adjacent land if there were too few people living within the originally selected county. Most of the PSUs were individual counties; from the ~3,100 counties in the U.S., 2,846 PSUs were formed. These PSUs were then selected at random through probability-proportion-to-size sampling. The Measure of Size (MOS) used to establish the size for weighting was derived from the U.S. 2000 Census and updated to account for large growth since that census. The MOS is not simply a raw population count, but a weighted average of estimated populations by race and Hispanic origin. This sampling methodology is done to ensure a mix of low-population and high-population counties exist in the NHANES county sample and that counties/groups with substantial number of populations selected for oversampling are more likely to be selected. Various other methodologies were applied to the PSU selection algorithm, such as the Ohlsson's method to minimize the chance a county selected in one year's sample had not been selected in a previous year's sample. The initial goal for number of people sampled in each PSU is 333, but is the goal was then re-adjusted per PSU to sample more people in PSUs that contain more people overall – especially more people representing demographics that will be oversampled.

The second stage consisted of a similar probability-proportion-to-size sampling as the county-level including a similar MOS algorithm, but instead it being used to select census blocks within the counties. While the general structure of the sampling methodology is similar between the first and second stage, the second stage has fewer added complexities – for example, it does not use the Ohlsson's method.

The third stage consisted of dwelling units (DU) or households. Within the selected census blocks, all DU were listed and of those, a subsample was selected. DU were selected with approximately equal probability; the necessary weighted sampling to obtain a nationally representative sample of households was conducted in the previous two stages and oversample demographic groups of interest was conducted in the first, second, and fourth stage. There were approximately equal numbers of households sampled in each segment selected.

Each sampled DU was then screened to determine if it was occupied, vacant, or if it were only occupied at certain times; only full-time occupied DU were retained in a list. From the list, a subsample of persons from each household were selected based on a combination of sex, age, race, Hispanic origin, and income – elements that constitute the 87 demographic subgroups of interest. The methodology also maximized the number of people per household sampled to increase the efficiency by minimizing the number of households required to achieve sufficient numbers of each of the demographic subgroups [70].

The expected breakdown of each year's data collection of NHANES is 15 PSUs will be selected. From that, 360 segments will be selected and 11,500 households within the segments will be screened. From those households, an expected 6,888 persons will be sampled, and 5,000 persons ultimately examined (figure 1). Because each NHANES cycle is two years, the estimated number of people examined in each cycle is 10,000.

This type of design is a departure from relying on random selection for representative selection; because random selection is an assumption made in many statistical procedures, special care needs to be taken in deciding how the data will be analyzed. While it is obviously essential to use survey weights to account for the design for calculating statistics relevant to the population simulated by the study (e.g. prevalence, averages), it is also important to account for survey design in regression models designed to report measures of disease association (e.g. relative risk). Even a well-adjusted regression model with sampling parameters included in the model as potential confounders can result in erroneous standard deviations if not accounting for the survey weights.[71]. Nevertheless, many studies use inadequate techniques for this type of complex survey design – some estimates are as high as 40% do not account for the sampling methodology [72]. Fortunately, each of the three major statistical analysis software has at least one method of accounting for complex survey design: R has package survey, STATA has svyset, and SAS has procedures such as SURVEYREG.

NHANES Data Collection

In the continuous NHANES, each year, mobile health centers are deployed to 15 different counties across the United States collecting data on approximately 5,000 people in total. The small number of counties collected per year means it cannot be used to make inferences on granular regions across the nation, but it can be used to establish more general regional differences (e.g. upper Midwest vs New England). The individuals are sampled initially for a house-hold screener which determines if any household members are eligible for the NHANES study. Eligible and consented individuals are then examined through combination of physical examinations, laboratory testing and household surveys. While the physical examinations and sample collection are performed within the mobile health centers, the health interviews are done in the participants' homes. The study team for each of these mobile health centers consists of a physician, medical and health technicians, and dietary and health interviewers. The survey data collected is directly entered in electronic form to decrease the likelihood of paperwork errors. After the data is collected and processed – including removal of personal identifiers – the data is made public to accommodate researchers. A subset of the data is withheld because it contains potentially identifiable information and/or highly private information; this withheld data can be accessed through an application process through the National Center for Health Statistics (NCHS) to access the data in specific locations to ensure the data cannot be used to identify persons or disseminated. Because of the wide nature of the term 'health' and variable funding, the data collected by NHANES has changed over time. Data has been collected through 2016, but it takes a substantial amount of time for collected data to be cleaned, de-identified and released.

The survey collected by NHANES includes demographic information, sexual behavior, drug use, two 24-hour food recalls and related dietary questions, cognitive and behavioral information, health conditions, etc. Though two 24-hour recalls were done, and Hepatitis E in the U.S. is thought to be most commonly foodborne, two 24-hour recalls are not enough for risk analysis. The only data potentially relevant to Hepatitis E that could be used was in the auxiliary questionnaire accompanying the 24-hour food recall – it includes shellfish consumption over the last 30 days and water source. The 2009-2010 cycle contained additional nutrition information section, but that has already been published in a previous study. The other data from the questionnaire used in this

study is primarily demographic – to characterize patterns of Hepatitis E – and health conditions – to examine individuals potentially at greater risk for infection or serious outcomes.

The physical and laboratory examination data includes blood pressure, body measurements, X-rays, oral examinations, serological testing, nutrient assays, urine tests, and a standard biochemistry panel. The exams include a multitude of chemicals and enzymes that are related to liver function: alanine aminotransferase (ALT**)**, alkaline phosphatase, aspartate aminotransferase (AST), total bilirubin**,** and platelets. Other data that will be included in the physical and laboratory examination data includes serological tests of Hepatitis E and other Hepatitis viruses – to examine for comorbidities.

Lab Testing of Hepatitis E

As of 2009, NHANES began serological testing of participants for Hepatitis E for the first time since it began continuously collecting data. Prior to this, the last time NHANES conducted serological testing for Hepatitis E was in NHANES III (1988-1994). As Hepatitis E is not a reportable disease and not routinely tested for even in the presence of liver ailments, it is difficult to assess the prevalence and impact of Hepatitis E outside of a study such as NHANES. Even NHANES cannot completely discern the prevalence and impact because Hepatitis E is often acute rather than chronic and neither the pathogenicity nor the virulence is well-defined in United States or other similar developed countries.

The NHANES III sera was analyzed with an in-house immunoassay, the DS-EIA-ANTI-HEV-G/M assay, and the WANTAI IgG and Western blot. Each of these assays are characterized as positive or negative; there is no indeterminant categorization. The recent NHANES cycles perform two types of serologic testing for Hepatitis E: DSI DS-EIA-ANTI-HEV-M – an IgM assay – and DSI DS-EIA-ANTI-HEV-G – an IgG assay. Both assays are produced by Diagnostics System (Soronno, Italy) (table 2). The IgG test has been verified to have a sensitivity of ~97.5% and a specificity of ~100**%** [73], but the IgM has not been as thoroughly characterized. While both are immunological assays, and

both can detect all genotypes of Hepatitis E, IgM detects if there has been a recent Hepatitis E infection, and IgG detects if one has ever had a Hepatitis E infection. While IgM theoretically provides a reasonable basis for the current prevalence of Hepatitis E, IgG cannot be the basis for current prevalence but can serve as an estimator for overall population-level risk. Using recent Hepatitis E infection is superior for finding proximal risk factors, but far fewer people were recently infected than ever being infected, diminishing the advantage of using the IgM. In contrast, IgG cannot identify proximal risk factors, but would be able to identify lifestyle risk factors assuming the relevant lifestyle risk factors did not change between the time of infection and the time of data collection. Due to the low numbers of people with an ongoing or recent infection of Hepatitis E, this study will use IgG when practical.

Data Set

Public use data from the Continuous NHANES, years 2009-2016 – four cycles – were included in the analysis. Variables were taken from HEV serology, dietary data total day 1, demographic, medical conditions, alcohol use, drug use, standard biochemistry profiles, and other hepatitis serology. The data was combined and cleaned using R 3.3.2.

For the Continuous NHANES years 2009-2016, we used parts of the restricted data set. This subset of data is withheld from public release due to its sensitive nature. Data considered to be sensitive includes personally-identifiable information – such as the location of their home – and information on vulnerable persons, such as information regarding one's pregnancies or information on youth – such as sexual behavior and drug use. To ensure the privacy of the participants, this data can only be accessed at certain sites. Any analysis must be done in the presence of a federal employee with appropriate clearances. These sites do not permit data to be taken in or taken out without review of an analyst. The access site we will use is the census restricted data center (RDC) site on UMN campus. While public documentation on the variables within the RDC is incomplete, both geographic and occupational data are included in the restricted dataset and will be used in the study. The data is combined and released to the researcher by a federal analyst, and the analysis will be done using R.

Only public use NHANES III, years 1988-1994, data was utilized. The primary NHANES III dataset was compiled in SAS and exported into R. Supplementary datasets examining Hepatitis E serology were appended to the primary dataset in R 3.3.2 where it was later cleaned and analyzed.

Seroprevalence and Risk Factors of Hepatitis E Virus in the United States

Synopsis

Previous estimates of HEV IgG seroprevalence in the United States sourced from the NHANES dataset ranged from 21% in 1988-1994 to 6% in 2009-2010. Since 2010, seroprevalence has not been reported. We evaluated the seroprevalence trend after 2010 and demographic patterns and tested for potential risk factors.

This study uses data collected from NHANES 2009-2016, specifically HEV IgG and IgM serology of non-institutionalized U.S. residents 6 years old and older. The weighted seroprevalence calculation for the U.S. population and demographic segments as well as age and sex-adjusted risk factor analysis were performed.

HEV IgG seroprevalence across 2009-2016 was 6.1% (95% CI: 5.6%, 7.0%) with approximately 1% of samples testing positive also for HEV IgM. HEV seropositivity was associated with race – especially high in non-Hispanic Asians (12.8%; 95% CI: 11.1%, 14.7%) – and being born outside of the United States (9.4%; 95% CI: 8.1%, 10.7%).

This study found the HEV seroprevalence in the United States to be largely stable over 2-year increments from 2009 through 2016. Additionally, we observed that non-Hispanic Asians were more likely to be HEV seropositive when compared to other races. Non-Hispanic Asians at higher risk should be accounted for in future Hepatitis E cohort or cross-sectional serological studies.

Introduction

Hepatitis E is an infection caused by the Hepatitis E virus (HEV) often presenting acutely as an asymptomatic or a non-specific febrile illness, but it also has potential for fulminant liver damage or chronic infection leading to liver-related complications and death, especially in immunocompromised people [25] [26] [27] [28]. There have been few studies examining prevalence of HEV in the United States because the virus was recently discovered in the U.S. in 1997 and has not been perceived as an urgent health crisis. Interest in evaluating the threat posed by Hepatitis E in the United States has increased since 2007, when chronic manifestations were first noted in immunocompromised individuals [74].

Two estimates of HEV seroprevalence from U.S. national studies document that HEV infection is not uncommon in the general population: 21% [75] and 6% [76]. These studies were published based on serologic tests collected by the National Health and Nutrition Examination Survey (NHANES) in years 1988-1994 and 2009-2010. The 1988-1994 seroprevalence study is of note because it offers a perspective of Hepatitis E during a time when it was not known to be endemic in the United States. Also, there appears to be a decline in the seroprevalence over the 15 years with no concerted interventions directed at the disease. If HEV infections are truly decreasing without intervention, reasons for the decline should be investigated. Has the seroprevalence truly declined sharply over the course of 15 years or are other external factors driving this change? One possible explanation could be the conflicting results from in serological tests used to evaluate HEV over the course of the last 15 years. In addition, it is important to better understand risk factors that may change over time that could impact Hepatitis E in the United States.

The risk factors and demographic patterns of HEV serostatus in the United States are poorly understood. Of two major studies examining HEV serostatus in the U.S., one study used NHANES sera from 1988-1994 [77], and the other study had examined demographic patterns using the 2009-2010 cycle but was not able to find many significant patterns [76]. Having three times as much timely data from new NHANES waves allows for examination of sub-groups with more precision; with the additional data collected since the 2009-2010 cycle, we can more precisely detect differences in

demographic and potential risk groups. As such, this analysis will pool the available cycles together to determine an average prevalence for the specific demographic group.

In the U.S. age has been the only demographic characteristic consistently associated with HEV infections. The HEV-age association has been extensively corroborated in studies conducted in other countries – while in part due to HEV seroprevalence tests detecting any infection in one's life, there has been indication that symptomatic HEV infections are more common in elderly populations [27] [25]. Other demographic characteristics including race/ethnicity, being born in the U.S., military service, education, and poverty has had conflicting or insufficient evidence [76] [75] [77] [73] and data from other countries has limited applicability.

Risk factors linked to specific transmission path such as waterborne, foodborne, and bloodborne, have not been established in the U.S. While waterborne infections are not typically thought of occurring in developed countries and has not been implicated as a risk factor in the U.S. [75] [76], HEV has been found in water in countries such as Switzerland and Canada [21] [78]. Similarly, shellfish consumption has not been specifically implicated as a risk factor in the U.S., but has been linked to infections in other developed countries [56] [79] [80]. Finally, while bloodborne transmission of HEV has not been clearly established as a major pathway [81], transmission of HEV through blood transfusion represents potential public health threat because chronic Hepatitis E can manifest in immunocompromised populations who receive blood transfusions [65]. Some countries, but not U.S., have begun to screen blood donors for HEV for this reason [82].

Each cycle of NHANES contains approximately 9000 participants sampled from 30 counties in the U.S. In each cycle, approximately 450 participants have been infected by Hepatitis E as shown by IgG test, and approximately 50 participants test IgM positive for recent infection. Thus, taken alone, each cycle can provide a reasonable estimate of HEV IgG seroprevalence, but the estimates for IgM seroprevalence would not be stable.

The goal of the study is to use the updated NHANES datasets to re-examine the prevalence, short-term trends, and lifetime risk factors of HEV in the U.S. population. A more detailed characterization of HEV in the United States may lead to increased awareness and testing in at-risk populations – such as those immunocompromised whose past infections may re-activate [1] – and those exhibiting Hepatitis E symptoms. A

downward trend over the 6-8 years covered could also indicate that the HEV prevalence truly declined since NHANES III.

Methods

Population

The data analyzed is from years 2009-2016 of NHANES. NHANES is conducted by the National Center for Health Statistics branch of the U.S. Center for Disease Control and Prevention. The source population is the entire United States, but the sample collected is clustered at multiple stages and stratified. The resultant sample contains weights to simulate the U.S. population. Thus, the results presented are representative of the entire non-institutionalized, civilian, resident U.S. population [69].

Variables

NHANES contains variables collected both via questionnaire and via laboratory sample testing. The majority of the variables assessed in this study are collected via questionnaire with the exception being the serological assay results for HEV infections. The variables analyzed by the study fall into two categories: 1) demographic variables characterizing variations in HEV seroprevalence across groups and 2) potential transmission pathways – blood transfusions, shellfish consumption, and tap water source.

Age was examined in strata of 10 year increments up to age 80 and above 80. Gender was characterized as male or female. Race/ethnicity was characterized once as Mexican American, other Hispanic, Non-Hispanic white, Non-Hispanic black and 'other race' (including multiracial) except when looking at 2011-2016 data that included non-Hispanic Asian as a distinct category. Birthplace was characterized as born in the U.S. (both states and territories) or born outside of the U.S. Additionally, immigrants were analyzed for years lived in the United States categorically as years =<5, 5 < years =<10, 10<years<=20, 20<years<=30, and years>30. Education was looked at as less than high school education, or high school education and greater. Poverty level was analyzed as below the poverty line, or at or above poverty line. Military personnel included anyone who had been a member of the Armed Forces of the U.S. including U.S. Army, Navy, Air Force, Marine Corps, Coast Guard, and members of the Reserves and National Guard whose units were activated.

Water source was analyzed as community supply, well or rain cistern, spring, don't drink tap water, and other. Shellfish consumption was characterized as shellfish eaten in the last 30 days, yes/no; we assumed if someone consumes shellfish in the last 30 days, they eat shellfish with some regularly. Blood transfusions were analyzed as ever having a blood transfusion or never having a blood transfusion.

The outcome variable used was seropositive as ascertained by the DSI DS-EIA-ANTI-HEV-G/M (DSI, Italy) assay. This assay is the only assay used to determine HEV serostatus in the continuous NHANES study. The findings reported are IgG, which is positive if someone has been infected. Results from the assay are characterized as positive or negative.

Statistical Analysis

Prevalence analysis

The average seroprevalence over all data available for years 2009-2016 and for individual cycles covering, 2009-2010, 2011-2012, 2013-2014, and 2015-2016 separately.

All data analysis was done using R 3.3.2 with Hmisc, dplyr, lme4 and survey libraries. Participants without HEV serology data were removed from data analysis. The primary outcome variable for seroprevalence was IgG positivity. NHANES uses a complex survey design that has clustering on multiple clusters, employs stratification, and oversamples certain demographic groups. These methods are essential for conducting the survey efficiently through narrowing the geographic scope from the entire U.S. to a small proportion of the counties, but they are a deviation from a simple random sample – the basis of many commonly used statistical techniques. Because NHANES does not use simple random sampling, specialized functions are required to obtain unbiased estimates and accurate standard error.

In R, the survey library contains functions to process survey design data and to properly analyze the survey design data post-processing. The svydesign function from the survey library was utilized to process the survey design data via applying weights and accounting for stratification and clustering. NHANES provided variables for clusters

(sdmvpsu), strata (sdmvstra) and weights (wtmec2yr)[2]. Once the survey weights, strata, and clusters were all accounted for by using the svydesign function, seroprevalence was calculated.

Prevalence estimates were done using the svyciprop function with the output of the svydesign function as one of the parameters.

Demographic Analysis

Like the prevalence analysis above, this was a descriptive analysis. HEV IgG seropositivity was used as the outcome variable and results was reported as the estimated percent of population seropositive with a 95% confidence interval. Identifying statistically significant differences was not the goal of the analysis; instead, it was to characterize the burden of HEV in subpopulations.

To accomplish this, the svydesign function and the svyciprop function were combined with a specialized subsetting function from the survey package, subset.survey.design. This permitted examination of seroprevalence in individual demographic groups. Performing the subsetting within the svyciprop was required because native R subsetting may delete important structural information, such as clusters or stratas, that are required to accurately estimate the standard deviation. A multivariate analysis containing these variables was conducted in the risk factor analysis, no multivariable model was run.

Because NHANES creates a representative sample based on gender/age/race characteristics, those variables can have accurate measures of seroprevalence calculated. Birthplace, education, and poverty index were not a part of the sampling algorithm NHANES so seroprevalence estimates and standard error may be distorted.

In addition to the prevalence estimates, risk ratios were obtained for most if the covariates in the study via the svyglm function with a quasibinomial family and log link. The primary outcome variable for seroprevalence was IgG positivity. Age and gender were included in all models as potential confounders.

[2] wtmec2yr can only be applied for analysis of a single 2-year cycle of NHANES. These weights were converted to 8-year weights (wtmec8yr) by dividing each cycle's weights by 4 for analysis of the full 4 cycles.

An additional multivariate analysis tested the interaction between birthplace and race/ethnicity. This analysis was done because there is an unequal distribution of HEV across the globe, and some racial groups outside of the U.S. may come from regions with greater burden than others.

Transmission Pathway Analysis-

Svyglm was used with a quasibinomial family and log link to conduct a multivariable logistic regression for risk factor analysis. First, each transmission pathway variable – blood transfusions, water, and shellfish – was tested independently in a model with age and gender. Then a multivariate model was conducted to account for potential confounding and better assess the potential transmission pathway variables. This multivariate model contained race/ethnicity and being born in the US as additional potential confounders.

Results

Seroprevalence in U.S. Population

The estimated national seroprevalence of HEV IgG averaged across 2009-2016 was 6.1% (95% CI: 5.6%, 7.0%). In 2009-2010, the seroprevalence was 5.96% (5.1%, 7.0%). In 2011-2012 the seroprevalence was 5.82% (4.6%, 7.0%). In 2013-2014 the seroprevalence was 4.6% (3.7%, 6.0%). In 2015-2016 the seroprevalence was 8.08% (7.0%, 10%). The seroprevalence for the short-term IgM assay varied between 0.5-1.5% each year with 2009-2015 overall estimate to be 1.02% (0.8%, 1.2%). Table 3 contains detailed HEV IgG and IgM results for each of the years and the overall estimate and indicates no short-term trend in seroprevalence exists.

Demographic Patterns of Seroprevalence

Table 4 shows a detailed breakdown of HEV IgG seroprevalence by demographic characteristics such as age, sex, race, birthplace, education level, and income. As expected, the IgG (lifetime seroprevalence) increases with age. Females had slightly higher seroprevalence than males. With respect to race, non-Hispanic Asians had significantly higher seroprevalence than any other ethnicity (12.8%) followed by non-

Hispanic whites (6.8%) the other racial groups – non-Hispanic black, Mexican-American, other Hispanic, and other all had between 2% and 5% seroprevalence. Those born outside the U.S. had a higher seroprevalence than those born inside the U.S. (9.4% to 5.5%). Little difference in seroprevalence was found different education groups and different income groups.

Table 5 shows the association between demographic variables and HEV IgG presented as risk ratios. Age and gender were not included because they were treated in each of the models as potential confounders. The only different conclusions from the seroprevalence table was military status was shown to provide a statistically significant protective effect (RR 0.69; 95% CI: 0.53,0.90) and years spent in US seem to be less significant.

Table 6 shows the interaction between racial/ethnic background and birthplace. Consistent with the simpler models, most racial/ethnic groups were at higher risk if born outside of the US. Notably, Mexican-Americans born in the US were less likely to have been infected with HEV compared to Non-Hispanic Whites born in the US (RR 0.36; 95% CI: 0.23,0.55); but Mexican-Americans born outside of the US were more likely to have been infected with HEV (RR 1.86; 95% CI: 1.45,2.37). Also notable is while the simpler analysis found Non-Hispanic Asians to be at higher risk than Non-Hispanic Whites, this model shows only foreign-born Non-Hispanic Asians were at greater risk.

Transmission Pathway Analysis

In addition to the demographic variables being expressed in table 5, the transmission pathway variables also are present. These analyses found non-significant results with respect all the transmission pathway variables: blood transfusion, shellfish consumption, and tap water source.

In the final multivariate model (table 7), the transmission pathway variables remained statistically insignificant. Being born outside of the U.S., race/ethnicity (Asians combined with other group in order to include years 2009-2010), and age were the only statistically significant variables in the final model.

Discussion

Seroprevalence and Demographic Estimates

The observed seroprevalence estimates was 6.1% and was similar to those found in the previous paper analyzing the continuous NHANES HEV serology – 6.0% [76]. The individual 2-year seroprevalence cycles are also similar, with highest seroprevalence in 2015-2016 and lowest seroprevalence in 2013-2014, 8.08% and 4.6% respectively. The highest seroprevalence occurring in the most recent cycle casts doubt on any continuing downward trend of HEV seroprevalence. The low IgM seroprevalence is suggestive that there did appear to be a significant upwards trend in HEV seroprevalence associated with age. This trend appeared steady and is consistent with other findings that infections occur at all ages [51]. It is unclear if the transmission pathways or genotype acquired differs in persons who are infected by HEV at different ages; a laboratory-based surveillance study found persons who acquired HEV abroad were much younger on average than persons who acquired HEV domestically [73].

We observed that non-Hispanic Asians had a significantly higher average seroprevalence (12.8% vs national average of 6.1%). Prior to 2011, non-Hispanic Asians were not included as a demographic group of interest. The reason for this observation is unclear but it remains significant after taking into consideration demographic variables of age and gender. Asians born outside of the U.S. seem to be driving the trend of higher seroprevalence for their racial group. However, certain racial groups appear to be less likely to have been infected by HEV than others. This is especially the case of Mexican Americans. This association is clearly not due to immigration from a low HEV-burden country as the foreign-born Mexican Americans were more likely to have been infected. In general, people born outside of the U.S. have higher seroprevalence, suggesting foreign acquired cases of HEV is a significant contributor to the overall national seroprevalence in the U.S.

Two additional demographic findings were unexpected; military status and immigrants having spent 5-10 year in the U.S. both were statistically significant in the models adjusting for age and gender. A risk association between HEV and military personnel would have been unsurprising if found to be a positive association, as this population can travel abroad for prolonged periods of time (i.e. deployed to developing countries).

However, the observed association was inverse. The higher prevalence in persons born outside of the U.S. who spent 5-10 years in the United States compared to those spending over 30 years was no longer statistically significant when including race in the model. This association could be explained by either type 1 error or variability in the country of origin of immigrants during different periods of time leading to a differential prevalence of HEV.

Transmission Pathway Analysis

In both the univariate and the multivariate analysis, the potential transmission pathway variables were not found to be associated with previous HEV infection. These findings are consistent with other studies in the US. While this study did not find evidence of an association between HEV, unsafe water, shellfish, and blood-born transmission, it does not exclude them as potential risk factors. Like the previous population-based analyses of HEV in the U.S., this study has the weaknesses of temporality and transient exposures (e.g. one may have a blood transfusion after having been infected by HEV, or one could have been infected by HEV while living in a place with an unsafe water source but has since moved). Notably history of blood transfusion does not have issues of being transient but was still null, suggesting that HEV is truly not often transmitted via blood in the United States.

Study Limitations

Though the additional data from years 2011-2016 improved our ability to detect smaller associations and temporal trends, the NHANES dataset did not substantially change, so structural limitations of NHANES still exist. One key limitation of the NHANES dataset is while they include two 24-hour food recalls, but for in-depth risk factor analysis, more 24-hour recalls are required. This precludes using NHANES to investigate the highly suspected pathway of pork consumption. In addition, HEV has a prolonged incubation period likely make 24-hour food recalls relevant.

The NHANES dataset, for reasons such as ease of data collection and preventing identifiability occasionally presents variables in categorical formats when a continuous variable may be more illuminating – for example, if time in U.S. were categorized continuously, we may be better able determine if the 5-10 year cohort is indeed at higher risk. Other categorizations were not as granular as needed; for example, non-Hispanic

Asians were not a racial categorization in all cycles, and birthplace being designated as "not in the U.S." is overly vague.

In addition, NHANES chooses a limited number of geographic regions per cycle, leading to a potential increased variability between cycles – which could be the source of the 2015-2016 cycle having higher seroprevalence than previous cycles. There may be other potential confounders that have not been collected as well. Finally, NHANES is a cross-sectional study; while many of the demographic variables are constant (e.g. race, birthplace), other variables (e.g. tap water source) may change over time. Thus, the ability to detect associations between some of the variables and HEV infection are hampered due to problems of temporality.

Future Directions

Continued research using HEV IgG and IgM seroprevalence is possible. The clearest future direction is a continuation of tracking national seroprevalence through NHANES for as long as NHANES includes the measures. This will allow for further examinations of temporal trends and potentially incorporate new variables introduced to NHANES.

Another potential direction would to design a cross-sectional study examining the potential link between pork consumption and HEV infection. Due to low seroprevalence in the general population and changing dietary patterns, it may be difficult to find an ideal population. Restricting to young-to-middle-aged adults would increase the prevalence in the sample. Further demographic studies, especially geographic analyses may further identify the optimal population to examine potential associations between pork consumption and HEV infection.

One potential problem of using a cross-sectional study to examine a relationship between HEV infection and pork consumption is immigrants from and travelers to highly endemic areas. The contribution of immigrants and travelers to average national seroprevalence is not known, though some effort has been made to characterize how many HEV infections were acquired abroad [73]. Further studies could examine the HEV serostatus of immigrants and people who travel abroad by time spent in different geographic regions.

Conclusion

This study examined HEV seroprevalence in the United States in years 2009-2016. We found that seroprevalence has not changed significantly in this time period and remains near 6%. Increasing age was consistently associated with being seropositive. While most potential risk factors (such as shellfish consumption and water source) were non-significant, we found a potential protective effect of being in the military and an increase in risk in immigrants who had spent 5-10 years in the United States. Finally, we saw a significantly higher seroprevalence in non-Hispanic Asians that was roughly twice the national average and significantly lower seroprevalence in all Hispanics. While age and immigration associations have hypothesized reasons, the cause for associations of military status and race/ethnicity remains unknown.

Comparison of Hepatitis E Immunological Assays using NHANES III Sera

Synopsis

In 2008, the seroprevalence of Hepatitis E from the years 1988-1994 was estimated to be ~21%; whereas current estimates of seroprevalence are as low as ~6%. While it is possible that the drop is real, the use of different assays in the two time periods may also contribute to the apparent drop in seroprevalence. This paper examines this the impact of differing Hepatitis E serologic tests and their impact on the reported seroprevalence over several decades in the United States.

This paper utilizes the NHANES III and continuous NHANES dataset, which encompasses the entire US population from 1988-1994 and 2009-2016, respectively. We examined the outcome of lifetime seroprevalence (IgG) with four assays – an in-house assay, the DSI ELISA kit, a Wantai ELISA and a Wantai Western Blot. Both standardization and survey weights were used to reconstitute the US population to estimate national seroprevalence. Agreement statistics were calculated to compare inter-test comparability. All analyses and seroprevalence estimations were conducted in R.

Using the in-house assay, the seroprevalence was 21%, but when using the more recently performed assay – DSI-EIA – the seroprevalence was 16%. We also observed significant differences in Cohen's Kappa and relative sensitivity and specificity between assays, demonstrating a lack of exchangeability of the tests when used for National serosurveys. Furthermore, we observed minimal differences between the seroprevalence calculated through survey weights versus standardization methods when the samples were randomly selected.

This study demonstrates that the decline in seroprevalence was real but likely overstated due to assay variability. This highlights a need to develop a gold standard assay or to standardize existing assays when conducting national seroepidemiologic surveys. The cause for the decline in Hepatitis E is still unclear.

Introduction

Hepatitis E (HEV) was first reported in the United States in 1997, but has been present in the country for an unknown length of time [15]. To characterize Hepatitis E exposure in the U.S. population as a whole sera was collected from its participants from 1988-1994 [83]. By examining serological data from NHANES III, a baseline Hepatitis E prevalence can be estimated for the early 90's. These data can then be used to establish a long-term trend of Hepatitis E seroprevalence. This trend data could better inform and identify potential factors affecting the changing seroprevalence on the U.S. population. One challenge is the lack of a standardized assay for Hepatitis E. This impacts the direct comparison of the data derived from the 1988-1994 sera and from more recently collected sera. The 1988-1994 sera were originally analyzed using an in-house assay. Further analyses of smaller subsets of the original 1988-1994 sera were conducted using a variety of HEV tests [84] [85]. The purpose of this study is to examine the seroprevalence of Hepatitis E from 1988-1994 using different assays; in addition, this study will examine the agreement between the assays.

While various serological assays such as recomWell, DS-EIA, Anti-Hepatitis E virus ELISA by Euroimmun, Wantai, and DiaPro are available for use, there is no gold standard for detecting a past Hepatitis E infection. Without a gold standard, multiple tests are used interchangeably. Currently there is a move away from using in-house assays [37] and less viable commercially available kits. In 2008, an in-house immunoassay was used [83]; the results of this assay were used in the paper that determined the seroprevalence was 21% despite the assay being uncharacterized [75]. In 2013 and 2016, the sera was re-analyzed – though the findings were not published – using the DS-EIA-ANTI-HEV-G/M assay [84] and the Wantai IgG and Western blot, respectively.

These tests have not been well-characterized in the literature and divergent results document either no difference [36] or a significant difference [41]. In the absence of a gold standard test, data cannot conclusively determine which test has superior sensitivity or specificity. We propose to compare an in-house assay with three commercially produced tests: DSI, Wantai ELISA and Wantai Western Blot. DSI is primarily used in the United States, and Wantai are commonly used in countries outside the United States.

It is especially important to compare the DSI assay from NHANES III to the DSI assay in the present study because previous studies found a large difference in the seroprevalence between the recent NHANES cycle that used the DSI assay and the NHANES III that used the in-house assay. If such a large difference truly exists, it would be difficult to explain without a substantial unknown risk factor or cohort effect – both potentials hold public health significance. As such, this study investigates the differences between the two assays used and the implications. If the difference in the tests can explain the difference between NHANES III and continuous NHANES, it will necessitate a discussion in the field regarding how reliable the IgG is for estimating seroprevalence. However, if the difference in tests cannot explain the difference between NHANES III and continuous NHANES, it would reveal that despite the aging population in the U.S., seroprevalence – and hence incidence – has been dropping.

Methods

Population-

The population being examined are primarily from NHANES III, conducted in years 1988-1994. To a lesser extent, data will be used from continuous NHANES years 2009-2014. Both NHANES studies were conducted by the National Center for Health Statistics branch of the U.S. Center for Disease Control and Prevention. The source population covered is the entire United States, but the sample collected is clustered at multiple stages and stratified. The resultant sample contains measures and weights to reconstitute a simulation of the U.S. population. Hence, the data collected by the NHANES is representative of the entire non-institutionalized, civilian, resident U.S. population [69]. To efficiently compare the Hepatitis E assays, the NHANES III sera were successively sub-setted (i.e. everyone tested by Wantai IgG were tested by all other assays; figure 2), so it is not known how well the sera tested by each assay represents the U.S. population.

Variables-

The variables examined in this study include the four different serological tests used to evaluate NHANES III sera for Hepatitis E antibodies. The DSI-EIA-ANTI-HEV-G assay was used to test continuous NHANES sera for Hepatitis E [40]. The tests being compared

are the 1) NHANES in-house immunoassay, 2) the DSI-EIA-ANTI-HEV-G assay (DSI, Italy), 3) the Wantai HEV-IgG ELISA (WANTAI, China), and 4) an anti-HEV Western Blot (WANTAI, China).

In-house IgG Immunoassay: This test was developed at the National Institute of Health specifically for the NHANES III study. The antigen used in the assay is a truncated 56 kDa recombinant HEV capsid protein (ORF2) expressed in insect cells. This, or an assay using similar mechanisms have been used in other studies including those examining Hepatitis E vaccine efficacy. The sera examined by this assay were tested once, and all tests were performed by the one technician. The sera was declared either positive or negative with no indeterminate classification [83]. This assay was used on 18,695 randomly selected sera from the original NHANES III sample. (Fig 2)

DSI-EIA-ANTI-HEV-G/M assay: This test was developed by Diagnostic Systems Italy The assay uses a mix of ORF2 and ORF3 recombinant antigens sourced from genotypes 1, 2, and 3 of HEV and grown from mouse cells?. [41]. The sera was declared either positive or negative with no indeterminate classification [40]. This assay was used on 5,966 randomly selected sera from those tested with the In-House assay. This is also the assay used in the continuous NHANES cycles. (Fig 2)

Wantai HEV-IgG ELISA: This test was developed in by Wantai and is sometimes known as Axiom. The assay uses the recombinant carboxy terminal of ORF2 sourced from genotype 1 for an antigen and grown in an unknown animal. [41] The sera was declared either positive or negative with no indeterminate classification [86]. This assay was used on 1,803 non-randomly selected sera from those tested with the DSI-EIA assay. (Fig 2)

Anti-HEV Western Blot: A western blot differs from the above assays in that it uses an electric pulse along with proteins to interact with your protein of interest to separate proteins by mass and charge. Through separating the proteins, one can identify individual proteins by purification or by using a reference. The details of the specific western blot used are not presented in the NHANES codebook. The sera was declared either positive or negative with no indeterminate classification [86]. This assay was used on the same 1,803 sera tested with the Wantai EIA assay. (Fig 2)

Statistical Analysis-

NHANES III Re-analysis & Comparison-

In order to compare the performance implications of using a given Hepatitis E assay, two sets of analysis will be done: 1) establish the seroprevalence from 1988-1994 under the different assays, 2) compare the assays used by calculating an agreement statistic. Data analysis will be done using R 3.3.2 with the Hmisc, dplyr, survey, epitools and CompareTests [87] libraries. Both data from NHANES III and continuous NHANES will be used. All four IgG assays done in NHANES III will be included: the in-house IgG assay, the DS-EIA-ANTI-HEV-G assay, the Wantai IgG, and the Western blot; however, the two Wantai assays were conducted on a non-random subset of the data, so they will not produce accurate seroprevalence estimates.

Seroprevalence Estimation:

The first analysis will be to compare the estimates of seroprevalence derived from using one of the four different IgG assays. There will be two methods of analysis to determine the seroprevalence of Hepatitis E in the NHANES III data: the first is to assume the survey weights are still valid and use code standard for complex survey design; the second is to disregard the survey weights because they may no longer be applicable to the subset of sera used in the assays and instead use standardization.

Seroprevalence Analysis using Survey Weights:

NHANES III data will be used as complex survey data, using packages to account for the clustering, stratification, etc. This analysis may produce inaccurate seroprevalence estimates for the NHANES III data – especially for the assays which only were performed on a small subset of the original data.

To establish a seroprevalence that is directly comparable to the continuous NHANES, we will use the svydesign function from the survey library in R. The svydesign function allows for the use of complex survey design data to obtain estimates from the population data. Like the continuous NHANES data, NHANES III data contains weights to make it representative of the U.S. population, but the NHANES III data does not contain the clusters or strata present in the continuous NHANES data. The results will be presented

in terms of the proportion of people testing positive for long-term exposure (using the IgG tests and the Western Blot).

Seroprevalence Analysis using Standardization:

NHANES III data will be standardized to a reference population using the ageadjust.direct function from the epitools library. Standardization was used to simulate the 2016 U.S. population on basis of age and race. While this method may not yield results directly pertinent to the current U.S. population or the U.S. population at the time of NHANES III, it will likely be more accurate in simulating how the different assays would apply to a theoretical population. This is especially pertinent to the assays which were only performed on a small subset of the NHANES III because it is not known how representative the subset was of the original complex survey methodology – i.e. the weights may no longer apply correctly and distort the outcomes.

Test Comparison:

In addition to estimating the seroprevalence, we will compare the performance of the different assays on the same individuals. Because all assays used nested subsets of the same NHANES III population (i.e. all persons tested with the Wantai assay were also tested by the in-house assay), we can directly compare the tests. One key advantage of doing test comparisons is they do not rely on the assumption that the tests were conducted on similar populations.

To determine the inter-assay reliability, we will use the CompareTests function from the CompareTests library in R. Apart from the in-house assay initially used and the DS-EIA-ANTI-HEV-G assay, two other comparable assays have been used on NHANES III sera: Wantai HEV-IgG ELISA and Anti-HEV Western Blot; the DS-EIA-ANTI-HEV-M assay is the only test that examines short-term exposure so will not be included in the analysis. Using CompareTests, a kappa coefficient will be calculated to determine how similarly the tests performed on a 1-1 basis. A κ of 0.8 or greater is considered a strong agreement [88]. In addition, we will calculate the sensitivity and specificity of the three assays assuming each other one is the gold standard.

This analysis has the advantage of bypassing the complex survey design issues seen in the seroprevalence analysis, such as incorrect weights. Because we assume there is no interaction between test performance and demographic groups and we are not concerned with population statistics, the weights and measures can be safely disregarded in this analysis. However, this means that the analysis cannot detect potential demographic biases in test performance and the inferences drawn would be difficult to apply to subsets of the population. Another drawback with this analysis plan is there is no established gold standard. We can say if a test produces on average more positive results than another test but not whether the test is more correct.

Results

Seroprevalence

Estimated Seroprevalence using Survey Weights

Table 8 shows the estimated U.S. seroprevalence using the NHANES III data and the NHANES 2009-2016 data calculated by using the recommended survey weights. The seroprevalence estimates by the in-house assay (20.8%) and the DS-EIA (16.5%) are statistically significantly different from one another. The Wantai and the Western blot were done on a non-random subset of the sera tested by the DS-EIA kit and hence cannot be used to infer the true U.S. seroprevalence.

Estimated Seroprevalence using Standardization

The estimated U.S. seroprevalence using the NHANES III data and the NHANES 2009-2016 data calculated by standardizing to an approximation of the 2016 U.S. population on age and race are shown in Table 9. The estimates of seroprevalence and conclusions align with the survey weighted methods above (~20% for the in-house and ~16% for the DS-EIA). For the estimates derived from random subsets of the NHANES III sera, the estimates differ by fractions of a percent; the estimates of the 2009-2016 change to a similar degree.

Estimates taken of non-random subsets did differ significantly between the survey weight method and the standardization method. The estimates changed by ~10% and were found to be statistically significant.

Relative Sensitivity & Specificity

The relative test sensitivities are displayed on Table 10. The in-house assay had a high sensitivity when assuming any of the other assays were the gold standard, but other tests had a wide range of sensitivities when tested against it as the gold standard (48-86%). Conversely, the DS-EIA assay had consistently low sensitivity (48-62%) and other tests had a high sensitivity when compared against it (96-97%).

Table 11 shows the relative specificity. When using any other assay as the gold standard, the in-house assay low specificity (38-79%), whereas the DS-EIA had a consistently high specificity (94-99%). The Wantai ELISA had variable specificity (42-88%), and the Wantai western blot had similarly variable specificity (54-85%).

These tables also provide information when their results are combined; for example, if we take the in-house EIA as the gold standard and the Wantai assay as the referent we find the sensitivity is 86% and the specificity is 88%; but when DS-EIA is the referent, we see a sensitivity of 48% and a specificity of 99%.

Kappa Scores

Table 12 shows the Kappa agreement score. All tests when compared to each other performed better than chance ($\kappa>0$); however, there still was significant disagreement. The DS-EIA and the Wantai had roughly similar agreement statistics when they were compared to the in-house assay ($\kappa\sim0.55$) [88]. The agreement statistic for the DS-EIA vs Wantai was weaker ($\kappa\sim0.35$). The strongest agreement statistic observed was when comparing the Wantai ELISA with the Wantai Western Blot ($\kappa\sim0.64$) – still fall short of a strong κ value.

Discussion

Discussion of seroprevalence estimates

We observed that when using the DS DSI-EIA applied to NHANES III sera, the seroprevalence of HEV IgG was 16.2%, a significant departure from the previous estimate using the in-house assay (20.9%) and trending towards, but still distant from modern DS DSI-EIA estimates of ~6%. This documents that the NHANES III reports of seroprevalence and the more recent reports of seroprevalence were only partially due to differences in assay methods.

While the two assays produced by Wantai cannot be used to determine seroprevalence due non-random sampling, the sensitivity and specificity analyses suggest the seroprevalence estimate would be between the DS DSI-EIA and the in-house assay. The substantial difference found between the assays' estimates of seroprevalence illustrates the necessity of more uniform testing of Hepatitis E.

Discussion of survey weights vs standardization

There were minimal differences between using survey weights of randomly subsetted data and standardizing the randomly subsetted data in terms of seroprevalence. This is suggestive that survey weights remain valid even when applied to a subset of the original dataset. The DS DSI-EIA was performed on less than 20% of the NHANES III participants (5,966 of 33,994 people were tested). This paper does not identify the point at which the survey weights become unreliable in their estimate.

Significant differences appeared between the two methods when the data was non-randomly subsetted. In this case, the standardization method resulted in more realistic results. The standardization method reported a highly unrealistic seroprevalence because selection was done based on test results; it remains to be seen how well standardization can estimate a parameter when the data is from a non-random subset of a larger dataset and the outcome is not a factor in the selection.

Discussion of Test differences

In this study, we found highly discordant results between four assays used to analyze the data. The two most similar tests were developed by the same company, Wantai, but

used different techniques; one was an ELISA and the other was a Western Blot. The in-house assay and the DSI DS-EIA were also ELISAs. ELISA assays may differ for many reasons, most notably by which part of which protein it is detecting, but also what threshold of signal qualifies as a positive result.

This is the first seroepidemiologic study to examine differences between commercial Hepatitis E kits. In previous studies, the population is either clinical subjects, of which a large subset is suspected to have HEV [36]; or a mix of blood donors and people suspected to have been infected with HEV [41]. This study examines a general population without respect to previous HEV infections. The general population has a far lower seroprevalence than people suspected to have HEV, as such the decrease in quality of the agreement statistic between this study is likely due to the test being designed as a clinical test, not an epidemiological screening tool. The poor performance in a low seroprevalence population is also indicative of poor specificity and poor positive predictive value; it can be inferred that tests with generally low specificity as compared to the other tests value sensitivity more than specificity. This appears to be the case with the Wantai products, as it has also been found in previous studies that they potentially give a higher rate of false positives and less false negatives [37]. This does not mean any of the assays examined in this paper are not suitable for clinical settings, rather more attention to specificity should be given when conducting population-level investigations. Of the tests done, the DS-EIA appeared to have the highest specificity, so may be the most appropriate in a population context, its high specificity is also supported by previous studies [41].

The Cohen's Kappa score of 0.35 between DS-EIA-ANTI-HEV-G and Wantai HEV-IgG ELISA represents significant disparities between the major commercial packages for Hepatitis E serology. In addition, the previous analysis of NHANES III sera significantly overestimated the seroprevalence of Hepatitis E. While the seroprevalence between NHANES III and the continuous NHANES have been found to more similar than previously reported, there still exists a large and unexplained difference between them.

Study Limitations

Due to the retrospective nature of this study, there were several limitations embedded in the data. One of the most significant limitations is only two of the major immunological assays were used. Several other relevant kits exist but were not used in testing the sera. The assays used were designed for clinical use, not epidemiologic surveys which may contribute to an inflated seroprevalence estimate. Further, some of the methodological details of the assays used were not reported, so changes to the assay protocol may have been made. The assays were conducted on consecutive subsetting of the original NHANES III data – while this study demonstrated that the subsetting at random was unlikely to significantly alter the estimates, a sample that had survey weights constructed for it would yield more accurate results. Finally, the subsetting used for the Wantai assay was non-random, so while sensitivity and specificity could be calculated, the seroprevalence estimates are severely biased.

Future Directions

Though we have provided further evidence that there has been a decline in Hepatitis E seroprevalence in the 15 years, we have not determined potential reasons for the decline. One possibility is previous age cohorts in the United States had higher seroprevalence due to a change in risk factors. While Hepatitis E is not thought of being waterborne in the United States, it could have been prior to public efforts to secure safe drinking water. The decreasing prevalence may be due to a decrease in waterborne disease in general in the United States [89]. A future study could compare declining trends of waterborne disease and foodborne disease across the 20th century to identify if either could explain the decline in Hepatitis E.

Further research is required in comparing the performance of the various Hepatitis E assays or perform a systematic review of the current literature pertaining on test performance in clinical and seroepidemiologic contexts. Ultimately, a gold standard for HEV infection must be established.

Conclusion

This study examined NHANES III serological samples tested with an in-house assay, a DSI kit, and two Wantai kits. We found significant deviations from the previously reported seroprevalence of ~21%, and now believe the seroprevalence in that time period is ~16%. While this change is significant, it is still far from the current seroprevalence

estimates of ~6%. The study found significant deviations in critical test attributes leading to the conclusion that in epidemiologic studies, the tests cannot be used interchangeably. We also determined there is minimal difference in parameters of a subsetted dataset in using standardization versus using native sampling weights when the subsets were randomly taken.

Liver Biochemistry in Persons Recently Infected with Hepatitis E Virus

Synopsis

The seroprevalence of Hepatitis E virus infection (HEV) in the United States is approximately 6%. While most of these cases are asymptomatic, the liver pathology of HEV is under-investigated. This study uses NHANES data containing over 10,000 subjects to assess the hypothesis that subclinical HEV can influence liver health. Two primary analyses were done: testing an association between HEV IgM and ALT – a marker of liver inflammation; and testing an association between HEV IgG and Fib-4 – a composite score reflecting potential liver fibrosis. These analyses were repeated in populations at risk for liver damage. The results found null results for the hypotheses tested. This is the first study examining predominantly subclinical effects of HEV infection in the United States, while studies have been conducted elsewhere, they have either been conducted in specific populations at risk for liver damage, or they focus on individual cases. The conclusions are suggestive that, in the general U.S. population, predominantly asymptomatic HEV infections do not contribute to the overall burden of liver disease and does not cause sustained liver inflammation.

Introduction

Hepatitis E is endemic to the United States, but its prevalence and impact remain unknown. Hepatitis E was discovered in the 1980's; and was only discovered in the United States in 1997. Between 1997 and 2008, Hepatitis E was generally not considered a major threat due to the typically asymptomatic self-resolving presentation of the only genotype found in the U.S. However, when the possibility of chronic infection by hepatitis was discovered in 2008, Hepatitis E was studied in more depth. Chronic Hepatitis E is typically defined as an active replication of the HEV infection lasting more than 3 months. A chronic infection is more likely to cause serious illness or death in immunocompromised persons than an acute infection. While the causal strain is endemic in the U.S., chronic Hepatitis E is primarily seen in Europe. Recent studies have reported Hepatitis E virus (HEV) to infect non-hepatic cells, such as neurons [90] and

have established the potential for a latent HEV infection to reactivate [91]. Further research is needed to better understand how HEV interacts with the body, especially the liver, outside of the context of what we recognize as HEV infection in terms of signs and symptoms.

Symptomatic cases of HEV infection typically present in an acute fashion – lasting approximately a month – and have different progressions depending on the genotype of the virus. The most prevalent genotypes worldwide are genotypes 1 and 3. Genotype 1 is found in resource-limited countries with unsecure water supplies whereas genotype 3 is found globally. Genotype 1 is more virulent than genotype 3, and can evolve into liver failure, especially in pregnant women. Genotype 3 infection can become chronic particularly in immunosuppressed individuals [63] [30]. Despite the difference in progression and virulence, the symptoms induced by both viral genotypes are similar. Symptoms of acute infections may include fever, anorexia, arthromyalgia, pruritus, dark urine, diarrhea, nausea, headache, abdominal pain, asthenia, vomiting, purpuric rash and jaundice; these symptoms are mainly related to hepatic inflammation and last from a few days to a month or more [25] [26] [27] [28]. In addition, both types of Hepatitis E are associated with increases in markers pertaining to liver inflammation; such as, serum alanine transaminase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase, and γ-glutamyltransferase [29] [30]. These signs and symptoms have been clearly described in the literature for symptomatic infections, but most infections in the U.S. are asymptomatic.

While HEV infection is commonly asymptomatic in the U.S., subclinical manifestations of HEV infection in asymptomatic cases can occur. As testing for Hepatitis E is not routine, detecting asymptomatic cases is unlikely in a clinical setting. Therefore, studies examining subclinical HEV infection either need to be specifically designed to detect subclinical infections or rely on large surveys that include HEV assays.

Previous studies have attempted to examine HEV in asymptomatic people. One study examining a population that had either dialysis or organ transplants in Argentina found a change in liver enzymes (i.e. AST and ALT) in seropositive HEV patients, but did not find symptoms associated with HEV [92]. A similar study conducted in southern Italy found much higher ALT levels in persons testing positive on IgM assay for HEV – while those testing positive on IgG did not have significant findings [93]. A study conducted in Germany followed ALT levels of 10 blood donors with asymptomatic HEV infection and

found half of them had elevated ALT levels [94]. Finally, a study conducted in Nepal found HIV positive persons who also had evidence of a prior HEV infection had far higher indicators of liver fibrosis as compared to HIV positive persons who tested negative for HEV [95].

This paper examines both potential short-term effects of Hepatitis E – liver inflammation – and potential chronic effects of Hepatitis E – liver fibrosis – using the National Health and Nutrition Examination Survey (NHANES). To determine liver inflammation, blood tests can be reliably used. The gold standard tests for examining liver fibrosis requires an examination of the liver, but NHANES does not conduct liver examinations directly. NHANES conducts a biochemistry panel that contains assays that can be used to estimate liver inflammation or damage – including tests measuring aspartate transaminase (AST), alanine transaminase (ALT), and platelet count. These tests can be used together to project whether someone has liver fibrosis or other forms of liver disease when using the Fib-4 score or the NAFLD score, respectively.

The NHANES data contains a biochemical assay containing the above measures of liver function. NHANES also measures antibodies IgG and IgM through a blood test; IgG detects whether a person has ever been infected with HEV whereas IgM detects whether a person has been infected with HEV within the recent past (approximately 6 months). As IgM indicates a recent infection, the data can be used to determine if there is a change in liver inflammation – indicated by an increase in serum ALT – following an infection of HEV. The IgM assay cannot distinguish between symptomatic HEV infections and asymptomatic HEV infections, but the low rate of symptomatic cases ensures the overwhelming majority of cases in NHANES are asymptomatic. So, by examining patients with positive IgM, we should be able to draw inferences for asymptomatic cases. This paper has two primary analyses: first examining IgM status in relation with ALT; second, using IgG and Fib-4 to determine if there is an increased risk of fibrosis in persons who had been infected with HEV. The latter analysis uses the IgG data rather than the IgM data because unlike inflammation, fibrosis is advanced damage to the liver that can become permanent.

Two sets of supplemental analyses were also conducted. The first supplemental analysis examined ALT in likely recent or active infections by examining participants with positive IgM but negative IgG. IgG antibodies takes longer to activate than IgM antibodies, hence examination of people with only IgM positive and not IgG positive sera

could indicate someone with an active or highly recent infection. The second set of supplemental analyses were examining ALT and Fib-4 among those with pre-existing liver conditions: other hepatic viruses, diabetes, heavy alcohol consumption, and unspecified liver conditions.

Methods

Population

The population being examined was from NHANES years 2009-2016. NHANES is conducted by the National Center for Health Statistics branch of the U.S. Center for Disease Control and Prevention. The source population is the entire United States, but the sample collected is clustered at multiple stages and stratified. The resultant sample contains weights to reconstitute a simulation of the U.S. population. In this way, the data collected by the study is representative of the entire non-institutionalized, civilian, resident U.S. population [69].

Variables

Analysis in General Population

The primary exposure variable in this study was presence or absence of HEV in serum ascertained by the DSI DS-EIA-ANTI-HEV-G/M assay. This assay was used to determine HEV infection in the continuous NHANES study. Results from the assay are characterized as positive or negative. This study compared IgG and IgM presence or absence to clinical laboratory results. A positive IgM titer represented recent infection and a positive IgM titer in the presence of a negative IgG titer represented an acutely recent infection. A positive IgG titer represented an infection in the past. These were used to assess inflammation analyses comparing ALT as an outcome for liver inflammation and Fib4 to measure liver fibrosis as the outcome.

Alanine aminotransferase (ALT) is found mainly in the liver's parenchymal cells. It catalyzes two steps of the alanine cycle – a process that cycles nutrients between the skeletal muscles and the liver. A normal range in female adults is ≤ 34 IU/L and ≤ 52 IU/L for male adults (although these values might vary); an elevated ALT level suggests damage to the liver – such as that seen in viral hepatitis.

The Fibrosis-4 (Fib-4) score estimates scarring of the liver. It is calculated by multiplying age by AST level, then dividing that by the platelet count multiplied by the square root of

the ALT level. For detecting moderate or advanced fibrosis, a cutoff of >1.0 gives a sensitivity of 64.5% and a specificity of 57.1%; for detecting advanced fibrosis, a cutoff of >1.45 gives a sensitivity of 70.0% and a specificity of 73.7% [96].

Potential confounders present in all analyses included were: age, gender, and race/ethnicity. Age was measured continuously up to age 80; people who were age 80 or greater were marked as being 80 for the analysis. Gender was characterized as male or female. Race/ethnicity was characterized as Mexican American, other Hispanic, Non-Hispanic white, Non-Hispanic black and other race (including multiracial).

Additional confounders include other liver conditions, alcohol consumption, injection drug use and/or diabetes. Persons with other liver conditions consists of a composite of persons with Hepatitis B infection, Hepatitis C infection, high NAFLD scores[3], or were self-reported in a questionnaire. Alcohol consumption was measured continuously as self-reported drinks per day. Diabetes condition was marked as present if the individual self-reported a doctor categorizing them as having diabetes or borderline diabetes. Injection drug use was also analyzed as self-report response of yes or no.

The outcome variables in the study include ALT measured continuously in IU/L and Fibrosis-4 measured binomially with a cut-off for a Fibrosis score of 1.45 which would strongly suggests advanced fibrosis [96].

Analyses in High Risk Population

Like the general population analysis, serum titer was ascertained by the DSI DS-EIA-ANTI-HEV-G/M assay. Potential confounders present in all analyses included were: age, gender, and race/ethnicity. Age was measured continuously up to age 80; people who were age 80 or greater were marked as being 80 for the analysis. Gender was characterized as male or female. Race/ethnicity was characterized once as Mexican American, other Hispanic, Non-Hispanic white, Non-Hispanic black and other race (including multiracial).

Variables used define the high-risk subgroup include persons with other liver conditions (including NAFLD or other Hepatitis infections), heavy alcohol consumption, injection

[3] The Non-Alcoholic Fatty Liver Disease (NAFLD) score – or NAFLD fibrosis score (NFS) – separates NAFLD patients with advanced fibrosis from those without advanced fibrosis. The score is calculated using age, hyperglycemia, body mass index, platelet count, albumin, AST and ALT. (Angulo et al., 2007) Clinically, NFS has been shown to be cost-effective through stratifying patients according to their level of need (Tapper, Hunink, Afdhal, Lai, & Sengupta, 2016).

drug use and/or diabetes. The variables included are similar to the general analysis but are coded categorically to define the subgroup.

A person would be defined as having other Hepatitis infections if either their Hepatitis B or C[4] tests were positive – the Hepatitis B serological test we used was the surface antigen test, and Hepatitis C infection was determined through RNA tests. Persons with other liver conditions were detected by questionnaire – analyzed as yes or no. We assessed probable NAFLD using the NAFLD fibrosis score binomially using 0.676 as the cutoff between a high NAFLD score and a medium/low NAFLD score. Heavy alcohol consumption was defined as having an average of 2 or more drinks per day or having had had more than 50 days in the last 12 months of drinking more than 4 and 5 drinks per day – for women and men, respectively. Diabetes condition was marked as present if the individual reported a doctor categorizing them as having diabetes or borderline diabetes. Injection drug use defined as self-report response of yes.

The outcome variables in this analysis are the same as that used in the above general population analysis.

Statistical Analysis

All analyses were done using “R” 3.3.2 with Hmisc, dplyr, lme4 and survey libraries. Participants without HEV serology data or biochemical exams were removed from analysis. There was no single primary outcome variable as both ALT and Fib-4 were used. NHANES uses a complex survey design that has multiple clusters, employs stratification, and oversamples certain demographic groups. These methods are essential for conducting the survey efficiently through narrowing the geographic scope from the entire U.S. to a small proportion of the counties, but they are a deviation from a simple random sample – the basis of many statistical techniques. As such, these methodological decisions were accounted for to obtain unbiased estimates and accurate standard deviations.

The survey library contains functions to process survey design data and to properly analyze the survey design data. NHANES provided variables for clusters (sdmvpsu),

[4] Hepatitis A not included because it is acute, meaning it has very low potential for a co-infection

strata (sdmvstra) and weights (wtmec2yr)[5]. Once the survey weights, strata, and clusters were all accounted for by using the svydesign function, the analyses were performed.

ALT Analysis in General Population

Multivariate linear regression was used to examine possible associations of HEV infection with liver inflammation. When assessing potential liver inflammation with ALT, the primary exposure variable for seropositivity was IgM positivity. ALT reported continuously to measure inflammation, and IgM status was analyzed binomially. An additional analysis examined IgM positivity in the presence of negative IgG. Age, race/ethnicity, gender, known liver conditions, alcohol consumption, diabetes, and injection drug use were included in both models as potential confounders.

Fib-4 Analysis in General Population

When assessing potential long-term liver damage with Fibrosis-4, the primary exposure variable for seropositivity was IgG. Fibrosis-4 was reported categorically with the cutoff for high chance of fibrosis being 1.45, and IgG status was analyzed binomially. Age, gender, known liver conditions, alcohol consumption, diabetes, and injection drug use were included as potential confounders. A linear model with similar parameters was also run to identify more subtle changes in Fib-4.

Analyses in High Risk Population

The primary inflammation analysis and the fibrosis analysis were repeated in persons at higher risk of liver disease. This high-risk group included people: with other liver conditions (including other hepatitis infections and NAFLD), with heavy alcohol consumption, who have used injected drugs, and people with diabetes. Because many of the confounders in the initial analyses were used to define the high-risk group, the only confounders in these models were age, race/ethnicity and gender.

[5] wtmec2yr can only be applied for analysis of a single 2-year cycle of NHANES. These weights were converted to 8-year weights (wtmec8yr) by dividing each cycle's weights by 4 for analysis of the full 4 cycles.

Results

Univariate Analysis

Demographics

The population demographics and other characteristics of the population analyzed with weights applied and shown on Table 13. Table 13 also contrasts the characteristics of those in the population testing positive on the HEV IgG assay to those testing negative on the assay. Notably, there were some substantial differences in this univariate estimation of HEV infections, most critical among them being those infected with HEV were on average ~20 years older than those who had not been infected. BMI was higher in the persons who were HEV IgG positive (29.1 vs 27.6). In addition, there were significant racial differences; non-Hispanic black persons and Hispanics who are not Mexican Americans were less likely to have been infected whereas non-Hispanic whites and other non-Hispanic persons were more likely to have been infected.

Clinical Signs

HEV IgG was also predictive for some clinical signs. People who tested positive for HEV IgG were also much more likely to test positive for HEV IgM (6.1% vs 0.7%). Additionally, Fib4 and NAFLD scores were elevated in the presence of positive HEV IgM (1.56 vs 1.03 and -1.19 vs -2.16 respectively); however, ALT and albumin levels were similar between people testing positive for HEV IgG and people testing negative for HEV IgG.

General Population Multivariate Analysis

Liver Inflammation (ALT)

Table 14 shows HEV IgM status tested against ALT. Testing positive for HEV IgM was associated with a non-statistically significant 0.22 decrease in ALT IU/L (95% CI: -4.17, 3.72). While HEV IgM was not statistically significant, reporting having a previous liver condition was significant (IU/L: 9.44; 95% CI: 6.90, 11.99). Other factors that increased ALT levels were being male (ALT IU/L: 8.46; 95% CI: 7.70, 9.21), having Hispanic heritage (Mexican Americans: ALT IU/L: 6.48; 95% CI: 4.49, 8.28 and Other Hispanic: ALT IU/L: 2.97; 95% CI: 1.45, 4.48), and alcohol consumption (ALT IU/L: 1.24 per average daily drink; 95% CI: 0.74, 1.70). Non-Hispanic Blacks appeared to have a lower-than-average ALT level (ALT IU/L: -2.07; 95% CI: -2.87, -1.28).

The analysis of HEV IgM positive where HEV IgG was negative is shown in table 15. This analysis found again no association between HEV IgM and ALT (ALT IU/L: -1.11; 95% CI: -5.53, 3.32). The factors that influenced ALT in table 14 were similarly present in table 15 with similar effect sizes.

Liver Fibrosis (Fib4)

Table 16 shows HEV IgG status tested against high Fib-4 status in the general population. There was a small, but statistically significant increase in the likelihood of having a high Fib-4 score associated with testing positive on HEV IgG (RR: 1.03; 95% CI: 1.01, 1.05). Other factors that increased risk of having a high Fib-4 score were being male (RR: 1.17; 95% CI: 1.05, 1.29), older age (RR: 1.08 per year; 95% CI: 1.08, 1.09), having other liver conditions (RR: 1.35; 95% CI: 1.24, 1.46), and high alcohol consumptions (RR: 1.04 per average daily drink; 95% CI: 1.02, 1.06). A similar model treating Fib-4 as a continuous variable did not find a statistically significant increase (0.05; 95% CI: -0.04, 0.14).

High-Risk Population Multivariate Analysis

Liver inflammation (ALT)

Table 17 shows HEV IgM status tested against ALT in the high-risk population – persons with other liver conditions (including NAFLD or other Hepatitis infections), heavy alcohol consumption, injection drug use and/or diabetes. In this population, HEV IgM was not associated with increased ALT (ALT IU/L: 2.87; 95% CI: -5.19, 10.94). The other results were broadly similar to those seen in the model run in the general population (Table 15) with males, and Mexican Americans having higher levels of ALT; ALT IU/L: 6.78; 95% CI: 5.40, 8.15 and ALT IU/L: 4.49; 95% CI: 2.06, 6.92 respectively. However, there were some differences; Hispanic people other than Mexican Americans no longer appeared to have higher ALT levels (ALT IU/L: 1.90; 95% CI: -0.64, 4.43) and there was a statistically significant decrease ALT IU/L as age increased (ALT IU/L: -0.12; 95% CI: -0.15, -0.08).

Liver Fibrosis (Fib4)

Table 18 shows HEV IgG status tested against Fib-4 in the high-risk population. In this population, HEV IgG was not associated with high Fib-4 score (RR: 1.02; 95% CI: 0.98, 1.06). The only variable found statistically significant was age (RR: 1.05; 95% CI: 1.05,

1.05). While being male was significant in the general population, it was no longer statistically significant in the high-risk population (RR: 1.05; 95% CI: 1.00, 1.11). Having had liver conditions and high alcohol consumption were not examined in this analysis because those variables were used to define this population. A linear multivariate analysis of Fib-4 also found a non-significant association with HEV IgG.

Discussion

Liver Inflammation (ALT) Findings

Associations between HEV IgM and ALT were not found. Though there were no associations between HEV IgM and ALT, the ALT analyses found gender, liver disease, and alcohol consumption to be associated with higher levels of ALT. In addition, race was associated with ALT; Hispanics had substantially higher ALT score than non-Hispanic whites, and non-Hispanic blacks having lower ALT than non-Hispanic whites. Age did not appear to influence ALT levels. These data demonstrate while some factors can increase liver inflammation levels, recent asymptomatic HEV infection does not.

Liver Fibrosis (Fib 4) Associations

We found a small association in both univariate and a log-scale multivariate model of HEV IgG and Fib-4 in the general population. The association in the univariate model was heavily confounded by age among other factors. The association in the log-scale multivariate model was small – having positive IgG increasing of a high Fib-4 score by approximately 3%. However, this association was not detected in the linear scale model or in any of the models using only the high-risk population. While these results are somewhat ambiguous, they clearly do not demonstrate a significant public health risk from seemingly asymptomatic HEV infections causing liver fibrosis. Apart from HEV IgG, other risk factors found for high Fib-4 score were not unusual – being male, being older, having liver disease, and higher alcohol consumption all contributed to a higher Fib-4 score.

Other studies

While the non-existent and weak associations between HEV infection and liver biomarkers are a departure from prior literature, this study was a cross-sectional study conducted in a healthy population. Though the studies conducted in Italy [93] and

Argentina [92] used the same kit as this study – DSI DS-EIA-ANTI-HEV-G/M – to detect HEV, these tests were conducted in clinical patients on hemodialysis and a combination of hemodialysis and organ transplant recipients, respectively. The study conducted in Nepal found a strong, positive association between HEV IgG and Fib-4, but it used a different kit, characterized the effects of a different genotype of HEV, and the study population was people infected with HIV [95]. Though HEV co-infection with HIV does not increase the rate of chronic cases as frequently as HEV infection in persons with solid organ transplantation, other studies have found synergistic effects in co-infected persons [97]. These studies show that HEV in certain subpopulations can have meaningful effects on the liver, but there is conflicting evidence whether asymptomatic HEV influences health on a general population level.

A study conducted in Germany was closest in scope and aims to our study [94]. While both studies examined many samples of asymptomatic, healthy people, Vollmer et al. utilized PCR to track active infections, whereas in our study we used IgM to identify recent infection (i.e. a more sensitive but far less specific approach). Vollmer et al. identified 10 persons with an active infection, but only 5 persons had substantial increases in ALT. While Vollmer demonstrated that acute liver inflammation is possible in asymptomatic infections of HEV, this study suggests the inflammation caused by acute, asymptomatic HEV occurs too infrequently and too briefly do be detected in those testing IgM positive.

Limitations & Future Directions

This study suggests HEV is unlikely a cause of liver inflammation or liver fibrosis in the U.S. general population. The null findings for inflammation could be explained by the IgM not being designed for population surveys; even with good specificity, the positive predictive value of IgM would be low due to the extremely low prevalence of positive IgM. This low PPV would dilute an effect of IgM on ALT. The weak/null IgG findings re-enforces the hypothesis that asymptomatic HEV does not have cause sustained liver inflammation. This conclusion is more certain than the conclusion of IgM because the prevalence of IgG is substantially higher than the prevalence of IgM, reducing the proportion of false positives. Despite not finding higher risk of liver inflammation or liver fibrosis in the high-risk population, this still warrants further investigation in immunocompromised individuals, as they are at greater risk of chronic HEV infection.

Conclusion

Overview of goals:

The goal of this dissertation was to quantify and characterize Hepatitis E virus (HEV) infections in the United States over the past 30 years but with a special focus on 2009-2016. In the first paper, I used NHANES 2009-2016 data to examine seroprevalence in both the general U.S. population and in specific demographic groups. In addition, I used this data to examine potential risk factors of Hepatitis E seropositivity identified in previous literature. In the second paper, I used NHANES III data to compare the performance of three different ELISA assays and one Western Blot used to diagnose HEV infection. In the third paper, I used NHANES 2009-2016 data to determine if subclinical HEV infection has acute or chronic liver pathology.

Summary of findings:

Paper 1 – Seroprevalence

We found HEV infections to be common, with seroprevalence in the general population being stable at around 6.1% from 2009-2016. There did appear to be a significant upwards trend in HEV seroprevalence associated with age. Non-Hispanic Asians had unexpectedly high average seroprevalence (12.8% vs the national average of 6.1%). Asians born outside of the U.S. seem to be driving the trend of higher HEV seroprevalence for their racial group. In general, people born outside of the U.S. have higher seroprevalence, suggesting foreign acquired cases of HEV is a significant contributor to the overall national seroprevalence in the U.S. In terms of the second goal, the study did not find evidence of an association between HEV, unsafe water use, shellfish, or blood-born transmission; but these cannot be excluded as potential risk factors.

Paper 2 – Test Comparison

When using the NHANES III data from 1988-1994 to compare the various diagnostic tests, we observed substantial differences between the assays' results. When using the DS DSI-EIA – the assay used in NHANES 2009-2016 –, the seroprevalence of HEV IgG was 16.2%, a significant departure from the previous estimate using the in-house assay (20.9%). Beyond the seroprevalence estimates, we found highly discordant results between four assays used to analyze the sera. The highest kappa agreement score was between two kits both produced WANTAI and was 0.64 – still not a strong kappa score.

Between two commonly used kits DSI EIA and WANTAI ELISA the kappa agreement score was only 0.35, which is typically thought of as a 'fair' kappa score.

Paper 3 – Subclinical Manifestations

When looking for subclinical manifestations of HEV infection, we found a small association in both univariate and a multivariate model of HEV and Fib-4 (an indirect measure of liver fibrosis). The association in the log-scale multivariate model was small – having positive HEV IgG increases the chance of having a high Fib-4 score by approximately 3%. This association was not detected, however, in the linear scale model. Associations between HEV IgM and ALT were not found. Apart from HEV IgG, other risk factors found for high Fib-4 score were not unusual – being male, being older, having liver disease, and higher alcohol consumption all contributed to a higher Fib-4 score. Like the HEV IgG and Fib-4 analysis, the IgM and ALT analysis found gender, liver disease, and alcohol consumption to be associated with higher levels of ALT. Major differences were that age was not influential, but race was; with Hispanics having substantially higher ALT score than non-Hispanic whites, and non-Hispanic blacks having lower ALT than non-Hispanic whites.

Conclusions

Paper 1 – Seroprevalence

Overall, this project has found that HEV infections are not rare in the United States. While initial estimates of ~21% from 1988-1994 sera overestimated seroprevalence, there still seemed to be a large decline to 16% and then 6% between 1988-1994 and 2009. However, between 2009 and 2016, seroprevalence estimates has remained stable at ~6%. This is indicative that sometime between 1994 and 2009, there was truly a dramatic trend downward that stabilized at 6%.

There were several demographic risk factors identified in this study, but no risk factors linked to transmission itself. We found that older people were more likely to have been infected and racial patterns such as non-Hispanic Asians to have over twice the average national seroprevalence. This does not necessarily mean they are at higher risk of contracting HEV in the U.S. especially because the association became non-significant

when accounting for people born outside the United States. Beyond non-Hispanic Asians specifically, we found evidence that a significant number of the people testing positive for HEV infection possibly acquired it abroad. In the case of Mexican Americans, we found those born outside the United States had a higher than national average seroprevalence – as expected – however, Mexican Americans in general had a lower than average seroprevalence. The reason for this is unknown but may be due to how cultural practices or geographic population distribution intersect with transmission pathways.

Paper 2 – Test Comparison

In both the seroprevalence estimates and the agreement statistics, there appear to be significant differences between the tests used to ascertain prior HEV infection. However, these assays were developed as clinical tests, not epidemiologic screening tools, and prior studies have found that when used in a clinical context, the tests are more comparable. The likely reason for this is that the general population has a far lower seroprevalence than people suspected to have HEV and the tests have been designed with sensitivity in mind. The poor performance in a low seroprevalence population is particularly indicative of poor specificity; in particular, it can be inferred that tests with generally low specificity as compared to the other tests value sensitivity more than specificity. Of the investigated tests, the DS-EIA consistently had the highest specificity when compared against other tests; this, combined with the low prevalence of HEV in the U.S. means DS-EIA would have the strongest positive predictive value, and may be the most appropriate in a population context.

Paper 3 – Subclinical Manifestations

This dissertation also found some evidence that subclinical HEV infections could contribute to liver fibrosis, but the associations were tenuous. There was no evidence of liver inflammation in persons recently infected with HEV. However, most of the cases detected in this study would likely be asymptomatic, so this finding does not contradict previous studies showing inflammation in patients with clinical cases of HEV infection. Overall, it appears that the impact of asymptomatic HEV infection and HEV infection in the general population is minimal, with only a slight, technical increase in risk found in liver fibrosis measured by an indirect score.

Future Directions:

Public Health Implications

Hepatitis E was not found to have a large public health impact in the United States. While the seroprevalence was non-trivial, we found little to no health impact to those infected. This evidence suggests, broad screening programs and vaccination programs are not warranted in the United States. This study did find that a significant number of people having been infected with HEV likely acquired it overseas, so a patient presenting with hepatitis symptoms who have recently been outside of the U.S. should be tested for HEV – especially because the genotypes present outside of the U.S. tend to be more virulent.

There are two major caveats: the focus on liver complications and the population examined. While HEV is commonly thought to be a virus that damages the liver, some research has shown it has a capacity to damage other organs. This study only examined the health impacts of HEV infection on the liver; it is possible that HEV in the general population has larger extra-hepatic impact than hepatic. This study also does not specifically examine the population most at risk for HEV infection complications – immunocompromised persons. As chronic HEV infection, HEV reactivation and other deleterious effects are primarily in the immunocompromised population, screening this population may still be a net benefit, but the tests used in this study rely on an intact immune system, so screening would likely need to be done with PCR tests. If HEV infection is detected, they should be further screened for liver damage and be considered for additional evaluation. Ribavirin has been shown to be an effective treatment in immunocompromised patients as well as those with intact immune systems [32].

Research Implications

While this study found current HEV seroprevalence in the U.S., identified demographic trends in HEV seroprevalence, clarified previous inconsistencies in the prevalence of HEV in the United States, and examined subclinical hepatic effects, there is still much to be learned about HEV infections in general and in the U.S. specifically. More research needs to be conducted on infection pathways in the U.S., diagnostic techniques, extra-hepatic manifestations, and long-term effect of viral infection.

This study was unable to find potential pathways for HEV transmission in the United States. This study examined water source, shellfish consumption, and blood transfusion. The largest potential risk factor that could not be assessed with NHANES with enough precision was meat consumption, specifically pork consumption. Finding pathways of infection for HEV would allow additional recommendations for those at risk for HEV complications. For these reasons, additional studies should examine potential pathways of infection of HEV.

This research also demonstrated that the many tests used to diagnose HEV do not have high agreement in an epidemiologic environment although previous studies showed mixed results of assay agreement in clinical context. Furthermore, NHANES did not use HEV RT-PCR diagnostic techniques on any of the samples. RT-PCR is an increasingly used diagnostic technique and has been applied to HEV. A major advantage of RT-PCR is the detection of virus itself with evidence of current replication, whereas serological assays only detects an immunological response – which continues to linger for a variable amount of time after the virus has been cleared. The inconsistencies between assays, lack of a gold standard, and new diagnostic tests entering the market all clearly demonstrate a need to conduct further research on the diagnostic techniques

This study did not examine potential extra-hepatic effects of HEV. It is possible that while typical symptomatic HEV infection primarily has hepatic effects, asymptomatic HEV instead could affect other body systems. In humans, HEV has been shown to impact different organs to varying degrees [74] [51]; with neurological [90], renal, pancreatic and hematological manifestations been reported. However, the validity of these associations and the overall impact in patients with asymptomatic, acute, and chronic HEV infection remains to be clarified. Further research needs to be done to establish the risk of these complications in each of these organ groups and infection stages.

None of these research questions can be adequately answered with the NHANES dataset because it does not collect enough data on risk factors, is limited to testing for HEV seropositivity with just one ELISA assay, and measure limited outcome variables. Due to the subclinical nature of HEV infections, both cohort and cross-sectional studies would be economically infeasible unless they were conducted as a component of another study that collected sera and included the relevant questions. In the absence of this capability, a case-control study would be ideal. A case-control study would be resilient against the relatively low incidence of HEV infections and would be able to

collect both sera and the data pertaining to infection pathways and routes. However, in the United States, HEV is not routinely screened for, sometimes even in the presence of otherwise unexplained hepatic disease, so the identification of cases would likely need to be done as a part of the study, rather than from a registry.

Final Thoughts

This study re-enforced previous findings that 1 in 16 people in the U.S. has been infected with HEV, but still no clear infection route has been identified. However, these findings may be disputed in the future if a similar study is conducted using a different serological assay – as another major finding of this study was a significant disagreement between different assays. Thus, more research needs to be done to establish a gold standard for current and past HEV infections. This study suggested that in the general U.S. population, there are few hepatic concerns from HEV manifestations; however, there have been studies showing HEV infections causing extra-hepatic effects, so there may still be some clinical concerns in the general population not identified in this study. Furthermore, it has been well established that HEV infection in immunocompromised populations can be chronically damaging. Overall, while this study did not find major public health implications in HEV infections, this study is not exhaustive in the identifying all the potential harms of HEV due to focusing on the general population and hepatic manifestations.

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Appendices

Table 1. Comparison of Hepatitis E between Developing Countries and Developed Countries

	Developing Countries	Developed Countries
Transmission	Waterborne	Foodborne
Highest Risk for Infection	Young Men	Middle-Aged Men
Elevated Mortality Risk in Pregnant Women	Yes	No
Chronic Manifestations	No	Yes
Potential for Large Outbreaks	Yes	No
Primary Genotypes	Genotype 1	Genotype 3

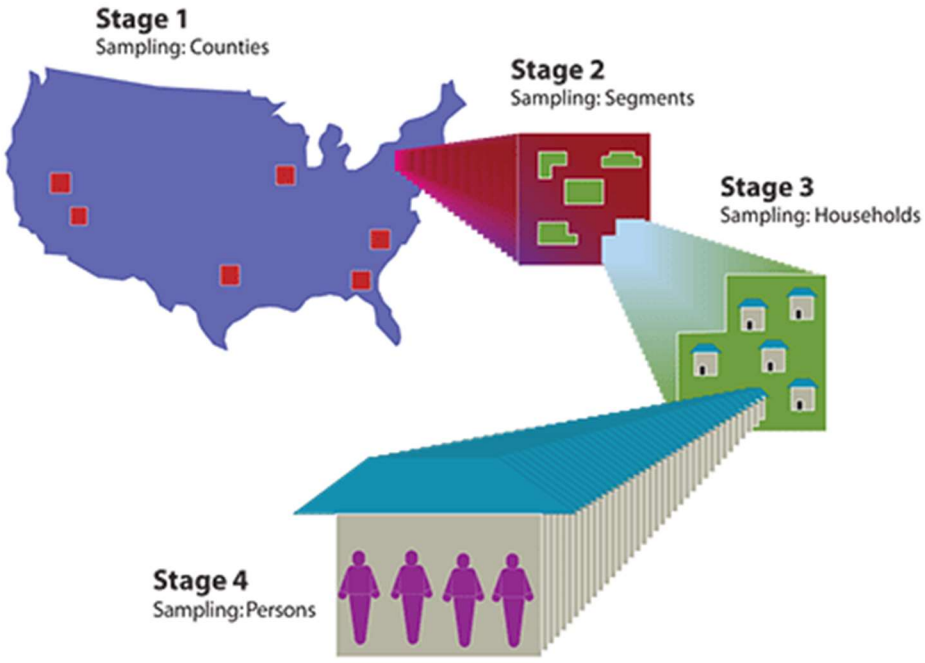

Figure 1. Sampling Methodology Summary. Source: CDC

Table 2. Hepatitis E Tests used in NHANES III and Continuous NHANES

	In House EIA	WANTAI HEV-IgG ELISA	Anti-HEV Western Blot	DS-EIA-ANTI-HEV-G/M
NHANES III	X (2008)	X (2016)	X (2016)	X (2013)
NHANES 2009-2010				X (2010)
NHANES 2011-2012				X (2012)
NHANES 2013-2014				X (2014)

Table 3. Seroprevalence of HEV in the United States 2009-2016

NHANES Cycle	IgG Assay Seroprevalence (95% CI)	IgM Assay Seroprevalence (95% CI)
2009-2010	6.00% (5.1%, 7.0%)	0.50% (0.3%, 0.8%)
2011-2012	5.80% (4.6%, 7.0%)	1.60% (1.2%, 2.2%)
2013-2014	4.60% (3.7%, 6.0%)	0.70% (0.3%, 1.1%)
2015-2016	8.10% (7.0%, 10.0%)	1.20% (0.9%, 2.2%)
Overall 2009-2016	6.10% (5.6%, 7.0%)	1.00% (0.8%, 1.2%)

Table 4. Seroprevalence of HEV in Demographic Groups in U.S. 2009-2016

	Seroprevalence Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Age in Years			
Below 20	0.50%	0.32%	0.74%
20-30	1.33%	0.96%	1.79%
30-40	2.91%	2.33%	3.59%
40-50	5.00%	4.05%	6.09%
50-60	8.08%	6.45%	9.96%
60-70	14.42%	12.22%	16.84%
70-80	17.08%	17.08%	17.08%
Above 80	18.48%	15.88%	21.32%
Gender			
Male	5.79%	5.27%	6.35%
Female	6.44%	5.69%	7.25%
Education			
High School Equiv or Above	7.12%	6.44%	7.85%
Less than High School	8.90%	7.57%	10.37%
Poverty Index			
Above Poverty Line	6.25%	5.62%	6.93%
Below Poverty Line	4.71%	3.81%	5.75%
*Race/Ethnicity**			
Non-Hispanic White	6.85%	5.93%	7.86%
Non-Hispanic Black	3.44%	2.92%	4.03%
Mexican American	4.37%	3.54%	5.33%
Other Hispanic	2.77%	1.97%	3.79%
Non-Hispanic Asian	12.82%	11.13%	14.67%
Non-Hispanic Other	4.42%	2.51%	7.14%
Military Status			
Is/Was in Military	6.21%	4.69%	8.04%
Never in Military	6.46%	5.78%	7.19%
Birthplace			
Inside U.S.	5.49%	4.91%	6.12%
Outside of U.S.	9.37%	8.14%	10.72%
*Years spent in U.S.***			
Less than 5	6.30%	4.52%	8.51%
5-10	9.06%	6.93%	11.58%
10-20	8.38%	6.54%	10.54%
20-30	10.04%	7.65%	12.88%
More than 30	12.46%	9.81%	15.51%

*Race/Ethnicity estimate 2011-2016 only because Asian race missing in 2009-2010

**Years spent in U.S. only assessed in persons born outside of the U.S.

Table 5. Summary of Individual Models of HEV infection Risk in U.S. (2009-2016) Adjusted for Age and Gender

	Risk Ratio Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Education Level			
High School or Above	1.00	REF	REF
Lower than High School	1.07	0.91	1.25
Poverty Index			
Above Poverty Level	1.00	REF	REF
Below Poverty Line	1.05	0.88	1.25
*Race**			
Non-Hispanic White	1.00	REF	REF
Non-Hispanic Asian	2.18	1.85	2.57
Non-Hispanic Black	0.68	0.56	0.82
Mexican American	1.16	0.94	1.43
Other Hispanic	0.63	0.46	0.87
Other	0.95	0.65	1.40
Military Status			
No	1.00	REF	REF
Yes	0.69	0.53	0.90
Birthplace			
Born in U.S.	1.00	REF	REF
Born outside of U.S.	1.75	1.49	2.06
*Years in U.S.***			
More than 30	1.00	REF	REF
Less than 5	1.22	0.84	1.76
5-10	1.65	1.25	2.2
10-20	1.29	0.98	1.69
20-30	1.22	0.93	1.60
Ever Blood Transfusion			
No	1	REF	REF
Yes	0.95	0.82	1.09
Recent Shellfish			
No	1.00	REF	REF
Yes	1.10	0.96	1.25
Tap Water Source			
Community Supply	1.00	REF	REF
Don't Drink Tap	1.05	0.53	2.08
Well or rain cistern	1.07	0.79	1.46
Spring	1.74	0.88	3.46

*Race/Ethnicity Analysis with 2011-2016 data due to Asian race classification missing in 2009-2010

**Years spent in U.S. only assessed in persons born outside of the U.S.

Table 6. Interaction of Birthplace and Race/Ethnicity in U.S. (2009-2016)

	Risk Ratio Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Intercept	0.01	0.00	0.01
Age in Years	1.05	1.05	1.05
Gender			
Female	1	REF	REF
Male	0.94	0.85	1.04
Race/Ethnicity & Birthplace			
US-Born Non-Hispanic White	1	REF	REF
US-Born Non-Hispanic Black	0.67	0.55	0.82
US-Born Non-Hispanic Asian	1.04	0.59	1.82
US-Born Mexican American	0.36	0.23	0.55
US-Born Other Hispanic	0.45	0.28	0.72
US Born Other Race	0.86	0.53	1.39
Foreign-Born Non-Hispanic White	1.41	0.90	2.23
Foreign-Born Non-Hispanic Black	0.83	0.43	1.62
Foreign-Born Non-Hispanic Asian	2.34	1.97	2.79
Foreign-Born Mexican American	1.86	1.45	2.37
Foreign-Born Other Hispanic	0.71	0.49	1.03
Foreign-Born Other Race	1.86	1.08	3.20

Table 7. Final Multivariate Model of Transmission Risk Factors of HEV in U.S. (2009-2016)

	Risk Ratio Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Intercept	0.00	0.00	0.01
Age in Years	1.05	1.04	1.05
Gender			
Female	1.00	REF	REF
Male	0.96	0.86	1.08
*Race/Ethnicity**			
Non-Hispanic White	1.00	REF	REF
Non-Hispanic Black	0.64	0.53	0.77
Mexican American	0.90	0.73	1.11
Other Hispanic	0.47	0.35	0.61
Other	1.15	0.97	1.37
Birthplace			
Born in US	1.00	REF	REF
Born outside of US	1.85	1.48	2.30
Tap Water Source			
Community Source	1.00	REF	REF
Don't drink tap water	0.98	0.82	1.18
Well or rain cistern	1.02	0.77	1.35
Spring	1.01	0.57	1.78
Shellfish Consumption			
No	1.00	REF	REF
Yes	1.07	0.94	1.22
Ever Blood Transfusion			
No	1.00	REF	REF
Yes	1.00	0.86	1.16

*Non-Hispanic Asian folded into “Other” category to include data from 2009-2010

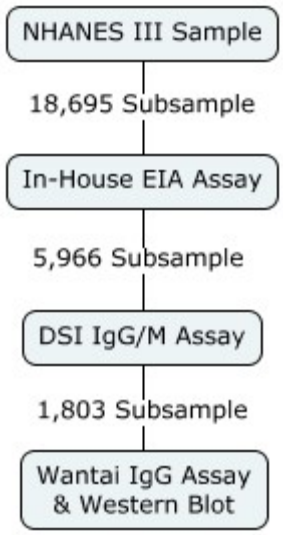

Figure 2. NHANES III Sera Subsampling Scheme

Table 8. HEV Seroprevalence in U.S. using NHANES Survey Weights (95% CI)

	NHANES III	NHANES 2009-2010	NHANES 2011-2012	NHANES 2013-2014	NHANES 2015-2016	NHANES 2009-2016
In House EIA	0.208 (0.199, 0.22)	NA	NA	NA	NA	NA
DS-EIA-ANTI-HEV-G	0.165 (0.150,0.18)	0.0596 (0.051,0.07)	0.0582 (0.046, 0.07)	0.046 (0.037, 0.06)	0.0808 (0.07, 0.10)	0.061 (0.056, 0.07)
WANTAI HEV-IgG ELISA*	0.734 (0.699,0.77)	NA	NA	NA	NA	NA
Anti-HEV Western Blot*	0.683 (0.647,0.72)	NA	NA	NA	NA	NA

*These were done on a non-random subset of DSI and hence should not be considered reliable estimates of national seroprevalence

Table 9. HEV Seroprevalence in U.S. using Standardization (95% CI)

	NHANES III	NHANES 2009-2016
In House EIA	0.209 (0.201, 0.217)	NA
DS-EIA-ANTI-HEV-G	0.162 (0.150, 0.174)	0.058 (0.055, 0.061)
WANTAI HEV-IgG ELISA*	0.629 (0.586, 0.677)	NA
Anti-HEV Western Blot*	0.588 (0.546, 0.636)	NA

*These were done on a non-random subset of DSI and hence should not be considered reliable estimates of national seroprevalence

Table 10. Comparative Sensitivity of HEV Assays from NHANES III

Gold Standard	In House EIA Sens (95% CI)	DS-EIA-ANTI-HEV-G Sens (95% CI)	WANTAI HEV-IgG ELISA Sens (95% CI)	Anti-HEV Western Blot Sens (95% CI)
In House EIA	**X**	**0.48 (0.47, 0.50)**	**0.86 (0.85, 0.87)**	**0.77 (0.75, 0.84)**
DS-EIA-ANTI-HEV-G	**0.96 (0.95, 0.97)**	**X**	**0.96 (0.95, 0.98)**	**0.97 (0.95, 0.98)**
WANTAI HEV-IgG ELISA	**0.97 (0.96, 0.98)**	**0.56 (0.53, 0.59)**	**X**	**0.86 (0.84, 0.87)**
Anti-HEV Western Blot	**0.96 (0.94, 0.97)**	**0.62 (0.59, 0.65)**	**0.94 (0.93, 0.95)**	**X**

Table 11. Comparative Specificity of HEV Assays from NHANES III

Gold Standard	In House EIA Spec (95% CI)	DS-EIA-ANTI-HEV-G Spec (95% CI)	WANTAI HEV-IgG ELISA Spec (95% CI)	Anti-HEV Western Blot Spec (95% CI)
In House EIA	**X**	**0.99 (0.98, 0.99)**	**0.88 (0.83, 0.91)**	**0.80 (0.76, 0.84)**
DS-EIA-ANTI-HEV-G	**0.79 (0.78, 0.80)**	**X**	**0.42 (0.40, 0.43)**	**0.54 (0.52, 0.56)**
WANTAI HEV-IgG ELISA	**0.54 (0.49, 0.58)**	**0.94 (0.92, 0.96)**	**X**	**0.85 (0.81, 0.88)**
Anti-HEV Western Blot	**0.38 (0.34, 0.42)**	**0.95 (0.93, 0.97)**	**0.66 (0.63, 0.69)**	**X**

Table 12. *Kappa Agreement of HEV Assays from NHANES III*

	In House EIA κ (95% CI)	DS-EIA-ANTI-HEV-G κ (95% CI)	WANTAI HEV-IgG ELISA κ (95% CI)	Anti-HEV Western Blot κ (95% CI)
In House EIA	**X**	**0.54 (0.52, 0.56)**	**0.59 (0.54, 0.63)**	**0.39 (0.35, 0.44)**
DS-EIA-ANTI-HEV-G	**X**	**X**	**0.35 (0.33, 0.38)**	**0.48 (0.45, 0.51)**
WANTAI HEV-IgG ELISA	**X**	**X**	**X**	**0.64 (0.61, 0.68)**
Anti-HEV Western Blot	**X**	**X**	**X**	**X**

Table 13. Demographic Characteristics of US Population from NHANES 2009-2016

	Overall Demographic Percent/Mean (95% CI)	HEV IgG Positive Percent/Mean (95% CI)	HEV IgG Negative Percent/Mean (95% CI)
Gender			
Female	51.2% (50.6%, 51.7%)	53.7% (50.9%, 56.5%)	51.0% (50.4%, 51.5%)
Male	48.8% (48.3%, 49.4%)	46.3% (43.5%, 49.1%)	49.0% (48.5%, 49.6%)
Age (In Years)	41.1 (40.5, 41.6)	59.3 (58.3, 60.2)	39.9 (39.3, 40.4)
Race/Ethnicity			
Mexican American	10.0% (7.8%, 12.2%)	7.9% (5.7%, 10.1%)	10.2% (8.0%, 12.3%)
Non-Hispanic Black	11.4% (9.6%, 13.3%)	6.4% (5.0%, 7.8%)	11.7% (9.8%, 13.6%)
Non-Hispanic White	64.3% (60.6%, 68.0%)	70.4% (66.9%, 73.9%)	63.9% (60.1%, 67.6%)
Other Race	8.1% (7.1%, 9.1%)	12.2% (9.9%, 14.5%)	7.8% (6.9%, 8.8%)
Other Hispanic	6.2% (4.9%, 7.5%)	3.2% (2.3%, 4.0%)	6.4% (5.1%, 7.7%)
Body Mass Index	27.7 (27.6, 27.9)	29.1 (28.6, 29.6)	27.6 (27.5, 27.8)
Family Poverty Index	2.86 (2.76, 2.95)	3.02 (2.89, 3.15)	2.85 (2.75, 2.95)
Hepatitis E IgG	6.1% (5.6%, 6.7%)	100.0%	0.0%
Hepatitis E IgM	1.0% (0.8%, 1.2%)	6.1% (4.3%, 7.9%)	0.7% (0.5%, 0.8%)
High Risk Group	22.0% (21.2%, 22.7%)	32.4% (29.3%, 35.4%)	21.3% (20.5%, 22.1%)
Other Hepatitis Infection	1.1% (0.9%, 1.2%)	1.7% (1.0%, 2.3%)	1.0% (0.9%, 1.2%)
Has Had Diabetes			
No	90.1% (89.6%, 90.7%)	82.3% (80.4%, 84.1%)	90.6% (90.1%, 91.2%)
Borderline	1.9% (1.6%, 2.1%)	2.8% (1.9%, 3.8%)	1.8% (1.6%, 2.0%)
Yes	8.0% (7.5%, 8.5%)	14.9% (13.1%, 16.7%)	7.5% (7.0%, 8.0%)

Ever Blood Transfusion			
Don't Know	0.9% (0.8%, 1.1%)	1.9% (1.0%, 2.7%)	0.9% (0.8%, 1.0%)
No	90.2% (89.6%, 90.7%)	81.6% (79.4%, 83.9%)	90.7% (90.2%, 91.3%)
Yes	8.9% (8.3%, 9.4%)	16.5% (14.3%, 18.6%)	8.4% (7.8%, 8.9%)
Ever Liver Condition			
No	96.5% (96.2%, 96.9%)	95.1% (93.2%, 97.0%)	96.6% (96.3%, 97.0%)
Yes	3.4% (3.0%, 3.7%)	4.8% (3.0%, 6.6%)	3.2% (2.9%, 3.6%)
Ever Injection Drug Use			
No	97.7% (97.3%, 98.1%)	98.4% (97.5%, 99.3%)	97.6% (97.2%, 98.1%)
Yes	2.2% (1.8%, 2.6%)	1.5% (0.6%, 2.3%)	2.3% (1.8%, 2.7%)
Average Daily Drink	0.59 (0.56, 0.62)	0.64 (0.51, 0.76)	0.59 (0.56, 0.62)
Fib4	1.06 (1.04, 1.08)	1.56 (1.50, 1.61)	1.03 (1.01, 1.05)
NAFLD	-2.10 (-2.14, -2.06)	-1.19 (-1.28, -1.10)	-2.16 (-2.20, -2.13)
ALT (IU/L)	24.8 (24.5, 25.1)	23.8 (23.0, 24.6)	24.8 (24.5, 25.2)
Albumin (g/L)	43.2 (43.1, 43.4)	42.7 (42.5, 42.9)	43.3 (43.2, 43.4)
AST (IU/L)	25.6 (25.3, 25.8)	26.0 (25.2, 26.8)	25.5 (25.3, 25.8)
Platelet (1k/uL)	242.4 (240.9, 243.9)	231.1 (227.4, 234.9)	243.1 (241.7, 244.6)

Table 14. Linear Model of ALT and HEV IgM Status in General Population from NHANES 2009-2016

	ALT IU/L Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Intercept	20.32	19.22	21.43
Gender			
Female	0	REF	REF
Male	8.46	7.70	9.21
Race/Ethnicity			
Non-Hispanic White	0	REF	REF
Non-Hispanic Black	-2.07	-2.87	-1.28
Mexican American	6.48	4.49	8.28
Other Hispanic	2.97	1.45	4.48
Other	0.58	-0.58	1.73
Age InYears	-0.01	-0.03	0.01
IgM Positive			
No	0	REF	REF
Yes	-0.22	-4.17	3.72
Ever Had Liver Condition			
No	0	REF	REF
Yes	9.44	6.90	11.99
Average Daily Drink	1.24	0.74	1.70
Diabetes Diagnosis			
No	0	REF	REF
Borderline	0.22	-2.15	2.60
Yes	0.04	-1.56	1.64
Ever Injection Drug Use			
No	0	REF	REF
Yes	4.17	-0.13	8.47

Table 15. Linear Model of ALT and Exclusively HEV IgM Status in General Population from NHANES 2009-2016

	ALT IU/L Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Intercept	20.33	19.22	21.44
Gender			
Female	0	REF	REF
Male	8.45	7.70	9.21
Race/Ethnicity			
Non-Hispanic White	0	REF	REF
Non-Hispanic Black	-2.07	-2.86	-1.28
Mexican American	6.48	4.69	8.28
Other Hispanic	2.97	1.45	4.49
Other	0.58	-0.58	1.74
Age In Years	-0.01	-0.03	0.01
Only IgM Positive			
No	0	REF	REF
Yes	-1.11	-5.53	3.32
Ever Had Liver Condition			
No	0	REF	REF
Yes	9.50	6.90	11.99
Avg Daily Drink	1.22	0.74	1.70
Diabetes Diagnosis			
No	0	REF	REF
Borderline	0.22	-2.15	2.60
Yes	0.04	-1.5	1.64
Ever Injection Drug Use			
No	0	REF	REF
Yes	4.18	-0.12	8.47

Table 16. Risk Ratio Model Liver Fibrosis and HEV IgG status in General Population from NHANES 2009-2016

	Risk Ratio Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Intercept	0.00	0.00	0.00
Gender			
Female	1	REF	REF
Male	1.17	1.05	1.29
Race/Ethnicity			
Non-Hispanic White	1	REF	REF
Non-Hispanic Black	0.95	0.77	1.19
Mexican American	0.89	0.80	0.99
Other Hispanic	0.94	0.84	1.06
Other	0.95	0.84	1.08
Age (In Years)	1.08	1.08	1.09
IgG Positive			
No	1	REF	REF
Yes	1.03	1.01	1.05
Ever Had Liver Condition			
No	1	REF	REF
Yes	1.35	1.24	1.46
Average Daily Drink	1.04	1.02	1.06
Diabetes Diagnosis			
No	1	REF	REF
Borderline	1.01	0.93	1.09
Yes	0.98	0.95	1.01
Ever Injection Drug Use			
No	1	REF	REF
Yes	1.21	0.98	1.48

Table 17. Linear Model of ALT and HEV IgM Status in High Risk Population from NHANES 2009-2016

	ALT IU/L Estimate	**95% CI Lower Estimate**	**95% CI Upper Estimate**
Intercept	30.36	28.26	32.45
Gender			
Female	0	REF	REF
Male	6.78	5.40	8.15
Race/Ethnicity			
Non-Hispanic White	0	REF	REF
Non-Hispanic Black	-2.27	-3.56	-0.98
Mexican American	4.49	2.06	6.92
Other Hispanic	1.90	-0.64	4.43
Other	3.10	-2.15	8.35
Age In Years	-0.12	-0.15	-0.08
IgM Positive			
No	0	REF	REF
Yes	2.87	-5.19	10.94

Table 18. Risk Ratio Model of Liver Fibrosis and HEV IgG status in High Risk Population from NHANES 2009-2016

	Risk Ratio Estimate	95% CI Lower Estimate	95% CI Upper Estimate
Intercept	0.02	0.01	0.02
Gender			
Female	1	REF	REF
Male	1.05	1.00	1.11
Race/Ethnicity			
Non-Hispanic White	1	REF	REF
Non-Hispanic Black	1.01	0.96	1.06
Mexican American	0.92	0.84	1.01
Other Hispanic	0.95	0.87	1.05
Other	0.93	0.84	1.04
Age In Years	1.05	1.05	1.05
IgG Positive			
No	1	REF	REF
Yes	1.02	0.98	1.06